The Promoting Activity on Human Megakaryocytopoiesis and Thrombopoiesis by Liquid Crystal-Related Compounds

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The liquid crystal compounds have a common structure with the cell membrane, having both a hydrophilic and hydrophobic residue, thus suggesting an affinity to the cell membrane. However, little information regarding a biological effect by liquid crystal compounds has been reported. In order to view the biological potential of liquid crystal compounds, the present study evaluated the *in vitro* human hematopoietic promoting effects by 18 liquid crystal-related compounds. In particular, these compounds are evaluated regarding their potential for platelet production from mature megakaryocytes by the culturing of CD34⁺ cells derived from normal human peripheral blood. Often, in the case of severe thrombocytopenia there is no choice but to perform a transfusion of platelet concentrates. Three of the tested compounds promoted megakaryocyte generation in the culture stimulated with thrombopoietin alone. In addition, two compounds led to a significant increase in CD42a⁺ particles which seemed to be platelets. At the same time, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF), that were used as a positive control for *in vitro* megakaryocytopoiesis and thrombopoiesis, resulted in a dramatic increase in the total number of cells; however, their promoting activity on *in vitro* hematopoiesis was almost at a similar level with the compounds. These results suggest that some liquid crystal-related compounds have a promoting effect on human thrombopoiesis, and that these compounds act with a different mechanism from either IL-3 or GM-CSF since the compounds specifically stimulated thrombopoiesis. The liquid crystal compounds may therefore be useful to develop a new functional medicine or a medical application.

Key words liquid crystal-related compound; megakaryocyte; platelet; CD34⁺ cell

In current cancer chemotherapy and radiotherapy, bone marrow depression occurring as a side effect frequently leads to a decrease in the function of hematopoiesis and the immune system in patients. Although some drugs that promote the production of leukocytes and erythrocytes are available for clinical use, there is no choice in severe thrombocytopenia except to provide a transfusion of platelet concentrates.¹⁾ However, frequent or continuous platelet transfusion with platelet concentrates causes a refractory state due to human leukocyte antigens incompatibility issues. Furthermore, a risk of viral infection accompanies this therapy. The well known mpl ligand thrombopoietin (TPO) has been shown to be the central physiological regulator of megakaryocytopoiesis and platelet production, and clinical trials of its effectiveness in thrombocytopenia have now been carried out in many countries.^{2,3)} However, the investigations of TPO have recently been hampered by the discovery of induced antibodies that neutralize TPO and cause thrombocytopenia.⁴⁾ New factors might be required to alleviate these problems. Although TPO-like activity has been discussed with regard to some low molecule peptide and non-peptide synthesis compounds, neither of the compounds under study has resulted in a clinical application as a platelet hematinic thus far.^{5–7)}

On the other hand, although many investigations regarding the characteristics and development of liquid crystal compounds have been studied in order to obtain superior compounds,⁸⁾ little information has been reported to date regarding their biological potential. The structure of an amphiphilic liquid crystal compound has a common structure with the

cell membrane in that each has both a hydrophilic and hydrophobic residue, thus suggesting an affinity to the cellular membrane. In addition, since liquid crystal compounds have the characteristics of orienting in either a magnetic or an electric field, their development may lead to either a new functional medicine or a medical application that can be regulated *in vivo* through the application of the appropriate external stimulation.

In our previous report, the effects of liquid crystal-related compounds on the megakaryocytopoiesis and thrombopoiesis were evaluated *in vitro* using CD34⁺ cells prepared from human placental and umbilical cord blood (CB).⁹⁾ About 20 kinds of compounds were tested to elucidate their effects on the clonal growth of CB CD34⁺ megakaryocytic progenitor cells (CFU-Meg) in plasma clot culture, with the result that one compound enhanced the clonal growth of CFU-Meg. However, the active compound did not promote thrombopoiesis from the megakaryocytes derived from the CB CD34⁺ cells. It is well known that *in vitro* thrombopoiesis is usually difficult to generate from megakaryocytes derived from CB.^{10,11)} In healthy individuals, the hematopoietic stem/progenitor cells circulating in the peripheral blood (PB) are more mature than their counterparts in bone marrow and CB.^{12,13)} In the present study, we therefore focused on the evaluation of platelet production by liquid crystal-related compounds from mature megakaryocytes *via* culturing in human PB.

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MATERIALS AND METHODS

Reagents Liquid crystal-related compounds used in the present study are shown in Table 1. No. 1, 3, 4, 5, 6 and 12 were obtained from Midori Kagaku Co., Ltd. (Tokyo, Japan). No. 15 was made by BDH Chemicals Ltd. (Atherstone, Warwickshire, U.K.) and was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The other compounds were synthesized by Japan Energy Corporation (Tokyo, Japan). Each compound was dissolved in dimethyl sulfoxide (DMSO).

Recombinant human thrombopoietin (TPO), human granulocyte-macrophage colony-stimulating factor (GM-CSF), human interleukin-3 (IL-3), IL-6 and IL-11 were purchased from Biosource (Tokyo, Japan). The amounts of each added are shown as follows; TPO, 50 ng/ml; GM-CSF, 1 ng/ml; IL-3, 100 ng/ml; IL-6, 50 ng/ml; IL-11, 100 ng/ml. The following fluorescence-labeled monoclonal antibodies were purchased from Beckman-Coulter-Immunotech (Marseille, France): fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (FITC-CD34), FITC-conjugated anti-human CD41 (FITC-CD41), FITC-conjugated anti-human CD42a (FITC-CD42a) and phycoerythrin (PE)-conjugated anti-human CD62p (PE-CD62p). Mouse FITC-immunoglobulin (Ig)G₁ and FITC-IgG_{2a} (Beckman-Coulter Immunotech) were used as isotype controls.

Purification of CD34 Positive Cells This study was ap-

proved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from normal human blood donors, PB was collected at the Aomori Red Cross Blood Center (Aomori, Japan). The buffy-coat was prepared from whole-blood by this center and was supplied to us. Light-density mononuclear PB cells were separated from the buffy coat by centrifugation for 30 min at $300\times g$ on a cushion of Lymphosepar I (1.077 g/ml; IBL, Fujioka, Japan) and washed three times with phosphate buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid. The CD34⁺ cell content was enriched by magnetic cell sorting (Miltenyi Biotec, Germany), performed according to the manufacturer's instructions except that the cells were applied to three magnetic bead columns to achieve high CD34⁺ cell purity. Finally, purified CD34⁺ cells were obtained from 0.1% of all cells present in the light density mononuclear cells. The expression of the CD34⁺ phenotypes in those cells, measured using a flow cytometer (EPICS-XL, Beckman-Coulter, Tokyo, Japan), was within the range of 88–95%.

Liquid Culture Peripheral blood CD34 positive cells ($2\text{--}3\times 10^4$ cells/ml in medium plus TPO, without liquid crystalline compounds; total volume 0.5 ml/well) were placed into 24-well plates (Falcon) and cultured in 2% human serum-containing IMDM supplemented with BIT9500 (StemCell Technologies, Vancouver, Canada). The cultures were incubated at 37 °C in a humidified atmosphere

Table 1. Structural Formula of the Liquid Crystal-Related Compounds

Sample number	Structural formula	Sample number	Structural formula
No. 1		No. 10	
No. 2		No. 11	
No. 3		No. 12	
No. 4		No. 13	
No. 5		No. 14	
No. 6		No. 15	
No. 7		No. 16	
No. 8		No. 17	
No. 9		No. 18	

containing 5% CO₂ for 14 d. On day 7, a sample solution or recombinant human cytokine were added. The same volume of DMSO was added to the culture containing TPO alone and it was used as the control culture. At the end of the culture, the cells were harvested and the number of viable cells was determined by trypan blue exclusion. The total number of megakaryocytes was calculated from the total number of cells harvested from the culture and the proportion of CD42⁺ among the harvested cells.

Pro-platelet Formation Analysis The pro-platelet-formed megakaryocytes were assayed by the modified plasma clot technique using platelet-poor human plasma as previously described.¹⁴⁾ This culture was plated onto 24-well cell culture plates at 0.3 ml/well, and then it was incubated at 37°C in a humidified atmosphere containing 5% CO₂. On day 12, each well was fixed twice for 15 min with acetone and methanol (2:1). The plates were air-dried overnight and stored at -20°C until they were stained. Prior to staining, the plates were thawed to room temperature; then, PBS containing 0.5% bovine serum albumin (PBS-B) was added to soften the clot. The solution was replaced with FITC-CD41 diluted 1:120 in PBS-B, and the plates were incubated at room temperature for 1 h, followed by a single wash with PBS-B. The nuclei were counterstained with propidium iodide (PI, 0.3 ng/ml, Sigma). After a final wash, the pro-platelet formed megakaryocytes were counted using fluorescence microscopy at 100× magnification (Olympus, Tokyo, Japan).

Immunological Marker Analysis The expression of cell-surface antigen was directly analyzed with immunofluorescence flow cytometry using single-staining with monoclonal antibody combinations including FITC-CD34 and FITC-CD42a. In brief, the cells were incubated with a saturated concentration of the relevant monoclonal antibodies for 20 min at room temperature, then they were washed by centrifugation (room temperature, 1200 rpm, 5 min) and added to Flow-Count standard beads (Beckman-Coulter, Inc., CA, U.S.A.) and analyzed with a flow cytometer. For each experiment, the negative control was provided by using irrelevant isotype-matched control monoclonal antibodies.

Platelet Functions After 14 d of culture, the cells were harvested and the number of viable cells was determined by trypan blue exclusion. The cell suspensions were added to adenosin 5'-diphosphate (ADP, 0.01 mM, Sigma) and then the cultures were incubated for 30 min at room temperature. After washing by centrifugation (room temperature, 1200 rpm, 5 min), the cells were incubated with a saturated concentration of the relevant monoclonal antibodies for 20 min at room temperature. The expression of surface antigen was directly analyzed by immunofluorescence flow cytometry using double-staining with monoclonal antibody combinations including FITC-CD42a and PE-CD62p.

Statistical Analysis A rejection test of outliers was performed in the results obtained by each experiment, and outliers were dismissed by a 5% level. Significant differences between the TPO alone control culture and the experimental groups were determined by Student's *t*-test.

RESULTS

Effects of Liquid Crystal-Related Compounds on the

Fig. 1. The Proportional Increase in the Total Number of Mononuclear Cells Generated in the Liquid Culture

Freshly prepared PB CD34⁺ cells were cultured in 2% human serum containing a liquid medium stimulated with TPO (50 ng/ml). On day 7, each compound (100 nM) was added. After an additional 7 d of culture, the cells were harvested from each culture and the viable cells were counted using trypan blue. Each value was shown by the mean $\pm$ S.D. of 3–8 separate experiments. * *p* < 0.05 compared to TPO alone by Student's *t*-test.

Growth of CD34⁺ Cell in Liquid Culture To evaluate the effect of liquid crystal-related compounds on megakaryocytopoiesis and thrombopoiesis, PB CD34⁺ cells were cultured stimulated by TPO alone. After 14 d of culture, the total generated cells were increased to 1.87-fold, 2.42-fold, 2.06-fold, and 2.71-fold respectively, from the TPO alone control culture by No. 2, No. 15, No. 16, and No. 17 (Fig. 1). By the addition of IL-3 and GM-CSF used as a positive control, an extreme promotion of cell growth was obtained with a 10.3- and 7.9-fold increase, respectively.

The total number of megakaryocytes was calculated from the total number of cells harvested from the culture and the proportion of CD42a⁺ among the harvested cells (Fig. 2). The compounds Nos. 14, 15, and 17 resulted in a significant increase of megakaryocytes to a 2.8-, 2.9- and 3.2-fold increase, respectively, from the TPO alone control culture. In the culture of IL-3 or GM-CSF, the total number of megakaryocytes increased to 2.4- and 1.9-fold from the TPO alone control culture.

The CD42⁺ particles, which seemed to be platelets and they were detected in the region of low forward scatter, were analyzed using a flow cytometer (Fig. 3). The compound No. 2 and No. 17 significantly amplified the production of CD42⁺ particles by 2.0-fold and 1.4-fold higher than the TPO alone control culture. At this time, the addition of IL-3 resulted in a 1.9-fold increase from the control.

Effect of Compounds on the Terminal Stage of Megakaryocytic Maturation To see the effect of the No. 2 and No. 7 compounds on the pro-platelet formation of mature megakaryocytes, the cytoplasmic extension containing all of the platelet organelles, including pro-platelet formatted megakaryocytes was scored on day 12 (Fig. 4A). No significant differences were observed in among the cultures with

Fig. 2. The Effects of Liquid Crystal-Related Compounds on Megakaryocytopoiesis Generated in the Culture by CD34⁺ Cells

After 14 d of culture, the cells were harvested and the expression of CD42a⁺ was analyzed using a flow cytometer. The total number of megakaryocytes was calculated from the total number of cells harvested from the culture and the proportion of CD42a⁺ in the harvested cells. Each value was shown by the mean $\pm$ S.D. of 3–8 separate experiments. * $p < 0.05$ compared to TPO alone by Student's *t*-test.

Fig. 3. The Effects of Liquid Crystal-Related Compounds on Thrombopoiesis Generated in the Culture by CD34⁺ Cells

After 14 d of culture, each medium was harvested and the number of platelet-like CD42a-positive particles was analyzed using a flow cytometer. Each value was shown by the mean $\pm$ S.D. of 3–8 separate experiments. * $p < 0.05$ compared to TPO alone by Student's *t*-test.

the TPO alone control culture and each treated compound, thus suggesting that each compound did not influence the rate of pro-platelet formation generated in mature megakaryocytes (Fig. 4B).

Functional Analysis for the Platelet Activation Generated in Liquid Culture The expression of CD34⁺ rate and CD42a⁺ rate in the cells freshly prepared from PB before culture were 89.0% and 1.5%, respectively (data not shown).

Fig. 4. Typical Pictures of Megakaryocytes and Pro-platelet Formation under the Fluorescence Microscope [A] and the Percentage of *in Vitro* Pro-platelet Formatted Megakaryocytes in Each Compound-Containing Culture [B]

Freshly purified PB CD34⁺ cells (1×10^3 cells/ml) were cultured in soft agar culture containing 10% human platelet-poor AB plasma with TPO (50 ng/ml) plus #2 (100 ng) or #17 (100 ng) for 12 d. Mature megakaryocytes and pro-platelets formation were stained with FITC-CD41 and PI. Values represent the mean $\pm$ S.D. of three wells from four separate experiments.

After culture, the CD34⁺ cells, relatively immature cells in hematopoiesis, dramatically decreased to 5–6% (data not shown), and CD42a⁺ cells, mature megakaryocytes, became the majority in the generated cells (64–70%) (Fig. 5). However, the rate of CD42a⁺ cells showed a similar level between the TPO alone control culture and the compound-added culture.

To see the function of CD42a⁺ particles, which seemed to be platelets, harvested from the culture supplemented with the No. 2 and No. 7 compounds, the expression of ADP-activated CD62p was analyzed (Fig. 5). The activation of CD62p was observed after the addition of ADP in the all samples. However, the expression on the surface of the CD42a⁺ particles showed no differences between the control and the compound-added culture.

DISCUSSION

The present study showed that several liquid crystal-related compounds significantly promoted the megakaryocytopoiesis and thrombopoiesis in a culture of thrombopoietin-stimulated human PB CD34⁺ cells. In particular, the promoting activity on thrombopoiesis by the compounds was almost similar to the level achieved with both IL-3 and GM-CSF that were used as a positive control on megakaryocytopoiesis and thrombopoiesis *in vitro* (Figs. 2, 3). None of the compounds influenced the rate of pro-platelet formation generated in mature megakaryocytes (Fig. 4). Although the precise mechanisms regarding the discrepancy is not clear based on only the present results, it is estimated that each megakary-

Fig. 5. The Results of a Flow Cytometry Analysis for the Markers of Platelet Activation

The platelets were generated from the cultures stimulated with the combinations of TPO plus each compound on day 14 of culture. The platelets activated with ADP (1 μ M) were treated with anti-human PE-CD62p monoclonal antibodies. The expression of each surface antigen was analyzed using a flow cytometer. Each value shows the CD62p positive percent gated in the CD42a⁺ cellular region. Typical cytograms are shown by three separate experiments.

ocyte can produce 10^3 to 5×10^3 platelets depending on their maturation level.¹⁵⁾ As a result, the two active compounds, No. 2 and No. 17, are not suggested to be involved the terminal stage of thrombopoiesis. In addition, a recent report regarding platelet production by megakaryocytes showed that the exact underlying mechanism of platelet release is still not well understood, and the release of platelets from mature megakaryocytes does not depend on the pro-platelet formation.¹⁶⁾ In this report, the author also mentioned the proto-platelet theory in which small number of short chains of platelets are released in the process of platelet birth from matured megakaryocytes.^{16–18)} Moreover, the IL-3 and GM-CSF resulted in a dramatically increased total number of cells (Fig. 1), but no increase in the total megakaryocytes. Although each CD62p level was relatively higher than the normal platelets due to manipulation of the flow cytometric analysis, the function of platelets generated from mature human megakaryocytes is almost identical to plasma-derived platelets (Fig. 5). Taken together, although more detailed studies are still required to elucidate the mechanisms that are involved in the relationship between the active compounds and mature megakaryocytes, the present results nevertheless suggest that the two compounds act by means of different mechanisms from both cytokines since they specifically stimulated thrombopoiesis, whereas neither compound showed any CB CD34⁺ CFU-Meg growth-promoting effect.⁹⁾ It is difficult to explain these different responses in terms of the structures and characteristics of these compounds. One possible reason is the differences in the target cells. There are considerable differences in the adhesion molecule expression and proliferation ability among the CD34⁺ cells contained in CB, bone marrow, granulocyte colony-stimulating factor-

mobilized PB and steady-state PB.^{19–21)} In particular, in a healthy individual, the hematopoietic stem/progenitor cells circulating in the PB are more mature than their counterparts in the bone marrow and CB.^{12,13)}

The well-known mpl ligand thrombopoietin, has been shown to be the central physiological regulator of megakaryocytopoiesis and platelet production, and clinical trials of its effectiveness in thrombocytopenia have now been carried out in many countries.^{2,3)} However, the investigations of TPO have recently been hampered by the discovery of induced antibodies that neutralize TPO and cause thrombocytopenia.⁴⁾ Therefore, the current investigations are now beginning to focus on antigen non-presenting molecules such as low molecular weight compounds. Inagaki *et al.* reported that a novel small molecule, JTZ-132 ((*S*)-7-hydroxyimino-3-(3-methyl-[1,2,4]oxadiazol-5-yl)-5-propyl-4,5,6,7-tetra-hydroxy-drobenzo[*c*]thiophene-1-sulfon-amide), increased the number of CFU-Meg in the culture of mouse bone marrow cells.⁵⁾ *In vivo* experiments in X-ray irradiation- or busulfan injection-induced myelosuppressed mice demonstrated that subcutaneously injected JTZ-132 at 30 mg/kg showed a significantly higher platelet number at the nadir and accelerated the platelet recovery without affecting the white blood cell number. Erickson-Millar *et al.* also reported that SB 394725, a small molecule thrombopoietin receptor agonists, is capable of activating TPO-specific signal transduction, proliferation, and differentiation responses similar to the responses and functions of the protein growth factor, TPO.⁶⁾ In addition, Nakamura *et al.* discovered a novel synthetic compound NIP-004 that displays the human TPO receptor (c-Mpl) agonist activity. This compound stimulates the proliferation or differentiation of human c-Mpl-expressing cells such as UT-7/TPO and human CD34⁺ cells.⁷⁾ In immunodeficient nonobese diabetic/Shi-scid, IL-2R γ (null) mice receiving transplants of CB-derived CD34⁺ cells, NIP-004 increased human megakaryoblasts, mature megakaryocytes, and circulating human platelets 6-fold, the latter being morphologically and functionally indistinguishable from normal human platelets. As shown in these reports, the developments regarding the novel molecules for thrombopoiesis such as non-peptidyl low molecular weight compounds are becoming active. Regarding the active compounds observed in the present study, compounds No. 2 and No. 17 were observed to have a flexible chain, a rigid aromatic core, and a hydrophilic unit. These amphiphilic features are thus thought to play an important role in the appearance of a lyotropic liquid crystalline phase. However, the other candidates, except for compounds No. 14 and No. 15, are also amphiphilic compounds with both a hydrophobic mesogenic group and a hydrophilic unit. However, no similar compounds have so far been reported, in addition, little information has so far been reported regarding the biological effect of liquid crystal compounds. The existence of other newly discovered pathways on the promotion of human megakaryocytopoiesis and thrombopoiesis can be expected. Furthermore, since a previous report has indicated that normal individuals have approximately 20–100 pg/ml TPO,²²⁾ a certain degree of synergistic action may be expected when an active compound alone is administered. We expect that the present study will lead to a new biological application of liquid crystal compounds, such as a functional medicine or a medical application that can be regulated by the appropriate

external stimulation such as that generated by a magnetic field or an electric field.

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