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Subtypes of inositol 1,4,5-trisphosphate receptor in human hematopoietic cell lines: dynamic aspects of their cell-type specific expression

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Abstract

Inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} signaling plays important roles in cellular responses to extracellular stimuli. We recently succeeded in cloning human counterparts of the three subtypes derived from separate genes. Using the cDNA sequences type-specific to these subtype receptors, we here analyzed the expression profile of IP_3R subtypes in stimulated and unstimulated human hematopoietic cell lines representing T cells, B cells, neutrophils, macrophages, erythrocytes and megakaryocytes. Northern and dot blot analysis showed that each IP_3R subtype is expressed differently in these cells and that the expression profile in each cell is dynamically changed upon stimuli which induce differentiation. Moreover, most of these cells were found to simultaneously express at least two different subtype receptors.

Key words: Cell differentiation; T cell; B cell; Intracellular calcium; Inositol 1,4,5-trisphosphate receptor; Hematopoiesis

1. Introduction

Most of cellular responses to various extracellular stimuli are coupled to G protein, which activates phospholipase C to hydrolize phosphatidylinositol 4,5-biphosphate to produce IP_3 and diacylglycerol. IP_3 binds to its specific intracellular receptors and releases Ca^{2+} from intracellular Ca^{2+} stores [1]. Generation of IP_3 also leads to Ca^{2+} influx via plasma membrane channels by an unidentified mechanism [2].

Progenitor cells in bone marrow proliferate and differentiate to multiple and distinct cell lineages [3]. Cell type-specific responses, such as cytokine production [4], differentiation to antibody-producing cells [5], chemotaxis [6], and phagocytosis [7], are initiated by stimulation of cell surface receptors followed by intracellular signal transduction: Ca^{2+} plays important roles in these processes. Phosphatidylinositol (PI) turnover [8], as well as the presence of IP_3 binding activity [9], have been reported for cells of bone marrow origin. Up-regulation of IP_3 binding activity in response to retinoic acid (RA), dimethylsulphoxide (DMSO) or vitamin D_3 have also been reported for HL-60 [10,11]. In these preceding studies, IP_3 receptor (IP_3R) was characterized only by IP_3 binding, Northern blot and polymerase chain reaction (PCR) analysis using sequence information derived from cDNA of murine IP_3R type 1 enriched in cerebellar Purkinje cells.

Molecular cloning studies have shown that there is a family of IP_3Rs. Full-length cDNAs for IP_3R subtypes, termed IP_3R type 1, 2 and 3, have been isolated [1,12–16]. We recently succeeded in cloning human counterparts of these IP_3R subtypes [17,30]. Using subtype-specific cDNA regions as hybridization probes, we have first found that all three subtypes of IP_3R are expressed in some lymphocyte cell lines [17]. In this study, we have examined the expression profiles of IP_3R subtypes in human hematopoietic cell lines of most lineages, and the dynamic change of the expression of these subtypes in response to stimuli which induce cell differentiation.

2. Materials and methods

2.1. Cells and cell cultures

The cell lines K562, HEL, CMK, ML-1, HL-60, KU812F and U937 were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 25 U/ml penicillin and 25 mg/ml streptomycin. HPB-ALL, Jurkat, HUT-78, Raji, Namalwa, RPMI1788, Jijoye and THP-1 were maintained in the above-described medium supplemented further with 50 mM 2-mercaptoethanol.

2.2. Induction of cell differentiation

K562 were treated with 50 mM hemin for 5 days. Cells were collected, broken open by freeze–thawing, and centrifuged at $200 \times g$ for 10 min and then at $10{,}000 \times g$ for 45 min. The hemoglobin content of the

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Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; PI, phosphatidylinositol; RA, ratinoic acid; DMSO, dimethylsulphoxide; PCR, polymerase chain reaction; TPA, 12-*o*-tetradecanoylphorbol-13-acetate; NBT, Nitroblue tetrazolium; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IL-3, interleukin-3; GM-CSF, granulocyte macrophage colony stimulating factor.

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supernatant was measured by scanning spectrophotometry [18,19]. CMK were treated with 10 ng/ml 12-*o*-tetradecanoylphorbol-13-acetate (TPA) for 2 days. Cell surface antigens were detected by immunofluorescence assay using mouse monoclonal antibodies Y2/51 and AN51 (Dako Japan, Japan) which recognize platelet membrane glycoprotein GP IIIa and platelet GP Ib [20,21], respectively. THP-1 and U937 were treated with 10 ng/ml TPA or 100 nM retinoic acid (RA) [22–24]. HL-60 cells were treated with 10 ng/ml TPA, 100 nM RA or 1.25% DMSO in serum-free medium [25–28]. α-Naphthylacetate esterase activity was determined by a chromogenic assay kit (Diagnostics) from Sigma (USA). Nitroblue tetrazolium (NBT) reduction was assayed as described [28]. The phagocytic activity of stimulated and unstimulated THP-1 was measured using sheep erythrocytes [28].

2.3. Northern and dot blot analysis

Total RNA was extracted and purified by the cesium chloride centrifugation method [29]. Poly(A)$^+$ RNA was purified with oligo(dT)-cellulose [29]. Poly(A)$^+$ RNA (3 μg/lane) was electrophoresed in 1% formaldehyde–agarose gels and transferred to nylon membranes (Hybond N$^+$, Amersham, UK). For dot blot analysis, Poly(A)$^+$ RNA was denatured and dotted to nylon membranes Hybond N$^+$ (0.5, 1 and 3 μg/dot). These blots were hybridized with the [^{32}P]cDNA probe specific to each subtype of IP_3R. Parts of the cDNAs, nucleotides 4833–5443 of mouse IP_3R type 1 in pBactS-Cl [1], nucleotides 4830–5669 of human IP_3R type 2 in pBluescript-IP_3R2, and nucleotides 4742–5694 of human IP_3R type 3 in pcDNAI-IP_3R3 [17], were cut-out by appropriate restriction enzymes and labeled by the random primer reaction. Our recent cloning of the full-length cDNA of the human type 1 receptor [30] indicates ca. 90% identity in the selected region between mouse and human IP_3R type 1. Blotted membranes were hybridized in a solution of 5 × SSPE (0.9 M NaCl, 0.8 M sodium phosphate, 5 mM EDTA, pH 7.7), 0.5% SDS, 5 × Denhardt's reagent and 20 μg/ml salmon sperm DNA at 65°C with 0.5 × 10^6 cpm/ml of probes, and washed in 0.2 × SSPE containing 0.1% SDS at 60, 55 and 65°C for type 1, type 2 and type 3 probes, respectively. The cDNA probe to human c-*myc* was cut-out by *Pst*I from pMYC7·4 [31]. For detection of human β-actin mRNA, the full coding region was amplified by PCR [32]. Radioactivity was detected with an image analyzer (BAS2000; Fuji Film, Japan).

3. Results and discussion

3.1. Subtype-specific probes for mRNA detection

Although the three IP_3R subtypes show high sequence homology, they have subtype-specific diverged regions patchily distributed throughout the entire stretch of their sequences [17]. We selected ca. 0.8-kb sequences immediately upstream of the transmembrane domains for Northern blot and dot blot hybridization.

The sequence identities among these regions of the three receptors are 60, 47 and 51%, for type 1/type 2, type 2/type 3 and type 3/type 1, respectively; we observed no cross-hybridization under the conditions employed in the following experiments.

3.2. IP_3R subtypes expressed in human hematopoietic cell lines

Northern blot hybridization analysis was carried out using the subtype-specific cDNA probes. Human hematopoietic cell lines with respect to their lineage, megakaryoblastic cell lines (HEL, CMK) [20,21,33], an erythroblastic cell line (K562) [19], a myeloblastic cell line (ML-1) [34], a promyelocytic cell line (HL-60), a monoblastic cell line (U937), a monocytic cell line (THP-1) [28], a basophilic cell line (KU812F) and lymphocytic cell lines (HPB-ALL, Jurkat, HUT-78, Raji, Namalwa, RPMI1788, Jijoye) were investigated.

Interestingly, most of the cell lines expressed more than one type of IP_3R simultaneously (Fig. 1). We observed a single band of about 10-kb in length that hybridized to IP_3R type 1 and type 3 probes. When the probe for IP_3R type 2 was used double bands of about 10-kb in length were detected as shown in HL-60 and Jurkat. This indicates the presence of different polyadenylation sites or splicing variants in IP_3R type 2 in hematopoietic cell lines. Similar results have been reported for the rodent IP_3R type 2 in peripheral tissues [12,13,35]. The presence of splicing variants has also been reported for IP_3R type 1 [36,37].

The expression profiles of the IP_3R subtypes were quite different among these cell lines. In the erythroblas-

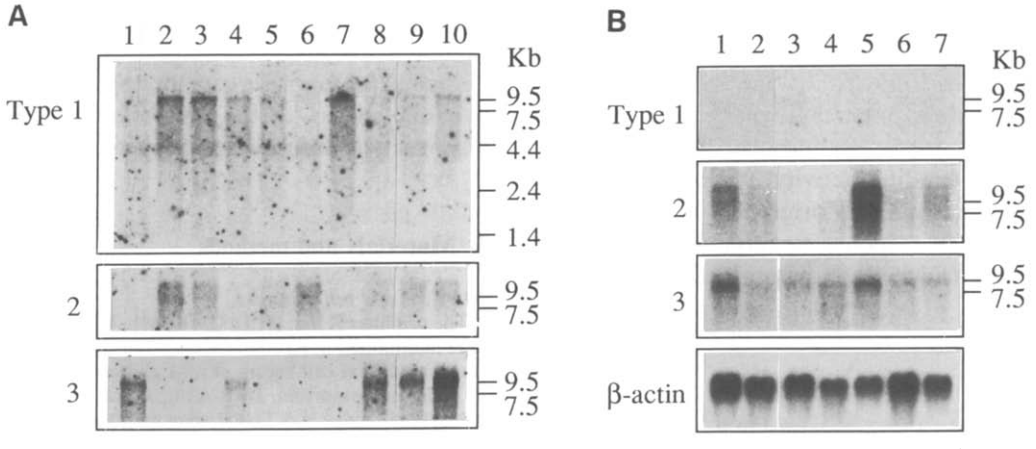

Fig. 1. Northern blot analysis of inositol 1,4,5-triphosphate receptor subtypes in human hematopoietic cell lines. Poly(A)$^+$ RNAs of K562 (lane 1), HEL (lane 2), CMK (lane 3), ML-1 (lane 4), HL-60 (lane 5), KU812F (lane 6), THP-1 (lane 7), U937 (lane 8), Jurkat (lane 9 in A, lane 2 in B), Raji (lane 10 in A, lane 4 in B), HPB-ALL (lane 1 in B), HUT-78 (lane 3 in B), Namalwa (lane 5 in B), RPMI1788 (lane 6 in B) and Jijoye (lane 7 in B) were electrophoresed, blotted on nylon membranes and detected by subtype-specific cDNA probes to nucleotides 4833–5443 of type 1 (top panel), nucleotides 4830–5669 of type 2 (middle) and nucreotides 4742–5694 of type 3 (bottom).

tic cell line K562, IP_3R type 3 was dominantly expressed, while type 1 was not detected. In the megakaryoblastic cell lines, HEL and CMK, the mRNAs of IP_3R types 1 and 2 were clearly detected but that of IP_3R type 3 was not. The T and B cell lines, Jurkat and Raji, displayed higher levels of expression of IP_3Rs types 2 and 3. As shown in Fig. 1B, we also analyzed the expression of IP_3R subtypes in several T cell lines (HPB-ALL, Jurkat, HUT-78) and B cell lines (Raji, Namalwa, RPMI1788 and Jijoye): each of these cell lines is thought to be at a different stage of differentiation. In these cell lines, with the exception of HUT-78, both IP_3R types 2 and 3 were detected while the type 1 receptor was only slightly detected under the experimental conditions used. This result agees with PCR analysis of murine thymus where mRNAs of only IP_3R types 2 and 3 were detected [35]. In HUT-78, a more mature T cell line than HPB-ALL and Jurkat, the type 3 receptor was dominant and tye type 2 receptor was hardly detected. The different behaviors of these T cell lines have been reported for interleukin-2 production [38,39] and intracellular Ca^{2+} increment after triggering formation of the T cell receptor–CD3 complex. It is reported that Ca^{2+} flux in HPB-ALL is almost solely dependent upon extracellular Ca^{2+} and is not coupled with PI turnover [40]. It is curious that the cell lines that express IP_3Rs type 2 and type 3 at the highest levels are the T cell lines. Thus, the functions of these IP_3R subtypes in T cells should be carefully studied with respect to regulation of their channel activity and subcellular localization, since there are reports by electrophysiology showing the presence of IP_3-induced Ca^{2+} influx [41,42]. In Jijoye, the most mature of the B cell lines described above, the ratio of the expression levels of IP_3R type 2: type 3 was considerably higher than those in RPMI1788 and Raji. No significant difference was observed in the Ca^{2+} response via surface IgM in murine B cells of either mature or immature stages in development, although the Ca^{2+} flux in the immature B cells was reported to be uncoupled with PI turnover. Moreover, the intracellular Ca^{2+} level at the resting stage of immature B lymphocytes is higher than that in mature cells [43]. Thus, such a difference in the expression of IP_3R subtypes in these B cell lines may be closely related to their characteristic intracellular Ca^{2+} metabolism. The subcellular localization of type 2 and type 3 receptors in T and B lymphocytes should be analyzed in detail by electron microscopy to determine whether the receptors are localized on the endoplasmic reticulum and/or plasma membrane. Recently, the presence of IP_3R in the plasma membrane of a B cell line has been suggested by electrophysiology [44].

The myeloblastic cell line, ML-1, and the promyelocytic cell line, HL-60, expressed different levels of IP_3R type 3, while mRNA levels of IP_3R types 1 and 2 were similar between these two cell lines. The expression profile of the basophilic cell line, KU812F, is characteristic

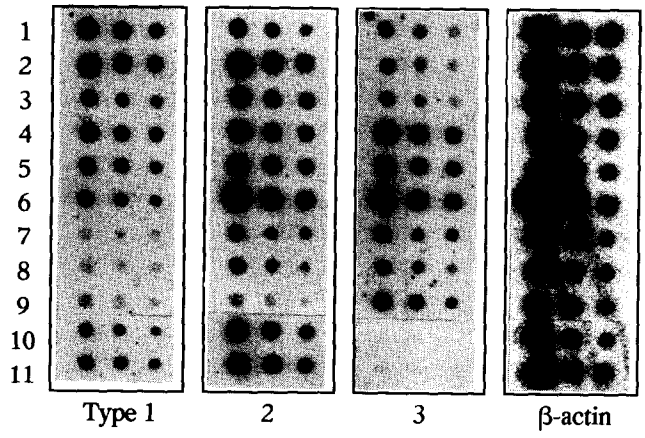

Fig. 2. Change in expression of inositol 1,4,5-trisphosphate receptor subtypes accompanied by the differentiation of human hematopoietic cell lines. Poly(A)$^+$ RNA from cell lines before and after differentiation was analyzed by dot blot analysis. The panels from left to right show autoradiograms of blots probed with IP_3R type 1, IP_3R type 2, IP_3R type 3 and β-actin, respectively. A single blotted membrane was used for reprobing. For each cell, 0.5, 1 and 3 μg of poly(A) RNA were blotted from right to left. THP-1 (lanes 1–3), U937 (lanes 4–6), K562 (lanes 7–9) and CMK (lanes 10,11) were stimulated with retionic acid (lanes 2,5), TPA (lanes 3,6,11), hemin (lane 8), and IL-3 plus GM-CSF (lane 9).

because it almost solely expresses the IP_3R type 2 message.

Although both THP-1 and U937 belong to monocyte/macrophage-like cell lines, the expression of IP_3R subtypes in these two cell lines was considerably different. IP_3R type 3 was dominantly expressed in U937, while it was not detected in THP-1. Conversely, the expression of IP_3R type 1 in THP-1 exceeded that in the U937 cell line more than ten times. U937 and THP-1 were established from histiocytic lymphoma cells and peripheral blood cells, respectively [45,46], and have different basal levels of phagocytic activity [23]. The specific expression of IP_3R subtypes in these two cell lines may reflect their different cellular functions.

3.3. *Changes in subtype-specific IP_3R expression accompanied by cell-differentiation*

The three monocytic cell lines, HL-60, THP-1 and U937, represent cells at different stages of differentiation [28] with distinct cellular functions regulated by Ca^{2+} mobilization more or less mediated by IP_3R [47,48]. The different expression profiles of the IP_3R subtypes in these three cell lines (Fig. 1) prompted us to examine if stimuli which induce cell differentiation and/or activation also cause a change in the expression profiles of the IP_3R subtypes. THP-1 and U937 cells were induced to macrophage-like cells by treatment with RA and TPA [22–24]. By microscopic observation of cellular α-naphthylacetate esterase activity, these cell lines in the unstimulated state showed weak but apparently positive staining, and the staining was intensified after stimulation (data not

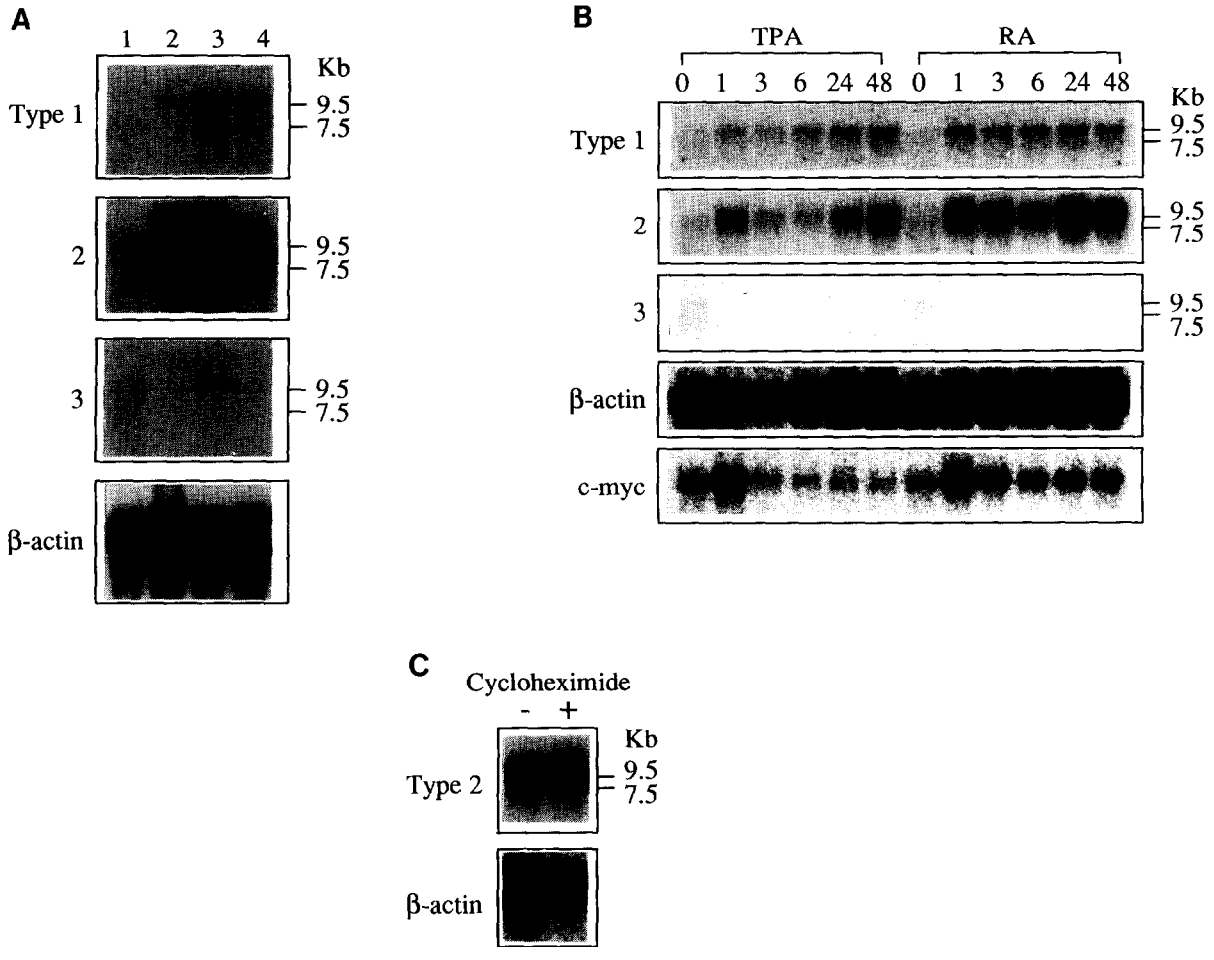

Fig. 3. Cell differentiation of HL-60 coupled with change in subtype expression of inositol 1,4,5-trisphosphate receptors. Poly(A)$^+$ RNA from unstimulated and stimulated HL-60 cells was analyzed by Northern blot analysis. (A) RNA from HL-60 unstimulated (lane 1), stimulated with DMSO for 5 days (lane 2), RA for 5 days (lane 3), TPA for 2 days (lane 4) were probed with type 1 probe (first panel), type 2 probe (second), type 3 probe (third) and β-actin probe (fourth). (B) Time-course of IP_3Rs expression upon differentiation. HL-60 cells were treated with TPA or RA for the indicated times. (C) Cells were also incubated for 48 h in the presence of TPA with or without 10 mg/ml cycloheximide. For controls, the blots were probed with β-actin and c-*myc*. A single blotted membrane was used for reprobing.

shown). The result indicates that unstimulated THP-1 and U937 cells already have some macrophage properties and differentiate in the presence of the inducer to a more mature state. The phagocytic activity of THP-1 against sheep erythrocytes increased from 48% to 70% following RA stimulation. Interestingly, when THP-1 cells were stimulated with RA, IP_3R types 1 and 2 were induced. TPA suppressed the expression of IP_3R type 1 in THP-1 and U937, although that of IP_3R type 2 was enhanced (Fig. 2).

In contrast to THP-1 and U937, HL-60 differentiates to both monocyte/macrophage and granulocytic lineages depending on the nature of the inducer [25–27]. TPA treatment leads HL-60 to become macrophage-like cells [25]. TPA-stimulated HL-60 cells with increased adherence and α-naphthylacetate esterase activity showed enhanced (IP_3R types 1 and 2) and decreased (IP_3R type 3) expression of IP_3Rs (Fig. 3A,B). These differentiated cells have been reported to have increased phagocytic activity as a result of induction of Fc receptors [22,49,50]. Our studies suggest that the Ca^{2+} signalling pathway to phagocytosis [7] may be mediated by IP_3R type 2, which is increased by stimuli leading cells to become phagocytes.

HL-60 cells were induced to granulocyte-like cells by sitmulation with DMSO or RA, as judged by NBT staining [25,27]. More than 80% of the cells were positive for NBT reduction by microscopic observation (data not shown). Like the TPA stimulation described above, DMSO and RA also induced the cells to express higher levels of IP_3R types 1 and 2, and suppressed the expression of IP_3R type 3 (Fig. 3A,B). It is remarkable that the IP_3R type 2 mRNA level was elevated more than ten times in DMSO- or RA-stimulated HL-60. This elevation by DMSO and RA stimulation exceeded that of TPA stimulation which leads the cells to become monocyte/macrophage like cells (Fig. 3A). Bradford et al. reported that functional IP_3R is induced in HL-60 differ-

entiated to the granulocytic lineage [10] and a single poly(A)$^+$ RNA species of IP_3R homologous to the murine Purkinje cell type IP_3R (type 1) is increased [11]. Our studies described here, using subtype-specific probes, have revealed that the molecular species of IP_3R elevated by the stimuli is mainly type 2. In human neutrophils, PI turnover and a transient increase in cytosolic free Ca^{2+} are required for migration and chemotaxis induced by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) [51]. Thus, it is of great interest to determine how the specific types of IP_3R are involved in regulation of the intracellular CA^{2+} mobilization associated with the behavior of monocyte/macrophage-like cells.

Interestingly, the increment of IP_3R mRNA levels is biphasic, with the first peak at 1 h and the second at 24–48 h, as shown in Fig. 3B. The induction of these receptors seems to be independent of protein synthesis since cycloheximide does not inhibit the induction (Fig. 3C). Many of the early response genes, such as c-*myc*, are those of transcriptional factors and therefore essential for the differentiation of HL-60 [52,53]. Fig. 3B indicates that the elevation of c-*myc* mRNA is somehow associated with the first phase elevation of mRNA of IP_3R types 2 and 3. It is widely accepted that intracellular Ca^{2+} is a major regulatory factor of cell differentiation [54]. Since cell differentiation is regulated by cascades of gene expression, it is important to know how and where the two IP_3R sybtypes are involved in those cascades.

CMK cells, which release platelet-like particles, are believed to be an ideal model for studying human megakaryocytopoiesis and for elucidating the biosynthetic process of megakaryocyte/platelet-specific proteins [21]. CMK cells were induced to cells having properties of mature megakaryocytes in the presence of TPA [20,21], as assessed by expression of the cell surface antigens, GPIb/GPIIIa (data not shown). Dot blot analysis showed that the levels of expression of IP_3R types 1 and 2 in these cells do not change before and after the stimulation. The result leads to the idea that platelets also retain these two IP_3R subtypes. Elevation of intracellular Ca^{2+} plays essential roles in the induction of platelet aggregation and secretion by thrombin, collagen, adenosine 5′-diphosphate, platelet activating factor and other agonists [55]. Thus, IP_3R types 1 and 2 may play essential roles in these platelet responses.

K562 cells are pluripotent stem cells [56] which are induced to the erythroid lineage by stimulation with hemin [19]. Unstimulated K562 cells expressed clearly detectable amounts of IP_3R types 2 and 3, and much lower amounts of IP_3R type 1. Induction of K562 cells with hemin, as assessed by the production of hemoglobin (data not shown), was found to be associated with the down-regulation of these two receptor subtypes. Stimulation with IL-3 plus GM-CSF is known to induce myeloid progenitors hematopoietic growth and maturation [57,58]. When K562 was treated with IL-3 and GM-CSF, mRNA synthesis for IP_3R type 2 was found to be prohibited while no apparant change was observed that of IP_3R types 1 and 3. The result is quite different from those described by Bradford et al. who reported that these two cytokines induce an increase in IP_3R as judged by an increased Ca^{2+} release from non-mitochondrial Ca^{2+} stores, Northern blot and PCR analysis using the IP_3R type 1 sequence [59]. The discrepancy remains to be examined.

In summary, the results presented in this work indicate that the three IP_3R subtypes are expressed cell-specifically in leukemia and lymphoma cell lines, and that the expression of these subtypes is specifically regulated during differentiation. These results suggest that the expression of the IP_3R subtypes changes dynamically in harmony with changes in cell functions. However, the molecular mechanisms of their regulation, in addition to the differences in the functions of each IP_3R, remain to be studied. Most of the cells studied here express two or three IP_3R subtypes simultaneously: it is also particularly interesting to examine whether they exist as homotetramers or heterotetramers, or whether the IP_3R subtypes are differently located in the cells.

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