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Expression of MAP3 kinase COT1 is up-regulated by 1,25-dihydroxyvitamin D_3 in parallel with activated c-jun during differentiation of human myeloid leukemia cells☆

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ABSTRACT

The role of MAP kinase pathways in 1,25-dihydroxyvitamin D_3 (1,25D)-induced differentiation of myeloid leukemia cells is well established, but the mechanisms by which 1,25D activates these pathways are not entirely clear. Following the finding that kinase suppressors of ras (KSR) 1 and 2 are directly regulated by 1,25D and participate in the monocytic differentiation process, we investigated if the COT1 oncogene (Tpl2 in the rat), known to interact with human KSR2 (hKSR2), is also involved in 1,25D-induced differentiation in leukemia cells. Here we report that the exposure of HL60 and U937 myeloid leukemia cells to 1,25D increases COT1 expression in a concentration-dependent manner. However, COT1 appears to have a differentiation-limiting role in these cells, as an exposure of HL60 and U937 cells to a pharmacological inhibitor of COT1 kinase activity, 4-(3-chloro-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino-3-cyano-[1–7]-naphthyridine, results in increased 1,25D-induced differentiation. These findings provide an additional insight into the 1,25D-regulation of MAPK pathways that contribute to monocytic differentiation process of myeloid leukemia cells.

1. Introduction

Clinically effective differentiation therapy in acute myeloid leukemia (AML) could supplement the current cytotoxic treatment regimens for this disease. All-*trans* retinoic acid (ATRA) has been demonstrated to induce terminal differentiation and hematological remission in 90% of acute promyelocytic leukemia (APL) patients [1]. Encouraged by this success, 1,25D and its analogs are being evaluated in preclinical studies as potential differentiation therapy agents for this disease, utilizing well-established cellular systems, such as the promyeloblastic leukemia HL60 and pro-monocytic leukemia U937 cells, to study the mechanistic basis of 1,25D-induced monocytic differentiation. Cell membrane-linked events have been implicated in this process, and one of the proteins that interact with membrane-associated proteins is encoded by the oncogene COT1 (Cancer Osaka Thyroid) [2], also known as Tpl-2 (Tumor Progression Locus 2) in the rat [3]. The COT1 gene is homologous to members of the MAP kinase kinase kinase (MAP3K) family, and has been reported to regulate the activity of several MAP kinase signaling pathways, including ERK, JNK and p38 [4–6]. For instance, the product of this gene can act in concert with Ras and Raf to activate MEK [7], and in mouse fibroblasts its over-expression can stimulate c-jun expression [8]. However, these pleiotrophic effects were reported primarily on the basis of studies in rodents, yet it is known that COT1 signaling is highly-cell type dependent [5]. Of interest to the vitamin D field, there is a non-consensus (nc) DR3 (single nucleotide nc) at +2829 of the COT1 gene [9], raising the possibility that 1,25D regulates this gene directly. In this study we investigated if COT1 participates in the process of 1,25D-induced differentiation in two human myeloid leukemia cell lines. Surprisingly, we found that while COT1 expression is up-regulated by 1,25D, it appears to have a negative effect on 1,25D-induced monocytic differentiation.

2. Materials and methods

2.1. Cell culture

HL60-G cells, derived from a patient with promyeloblastic leukemia [10], and U937 cells, derived from human histiocytic lymphoma [11], were cultured in suspension as previously described [12].

2.2. Reagents and antibodies

1,25D was a kind gift from Dr. Milan Uskokovic (Bioxell, Nutley, NJ). The antibodies for Western blotting studies were obtained from

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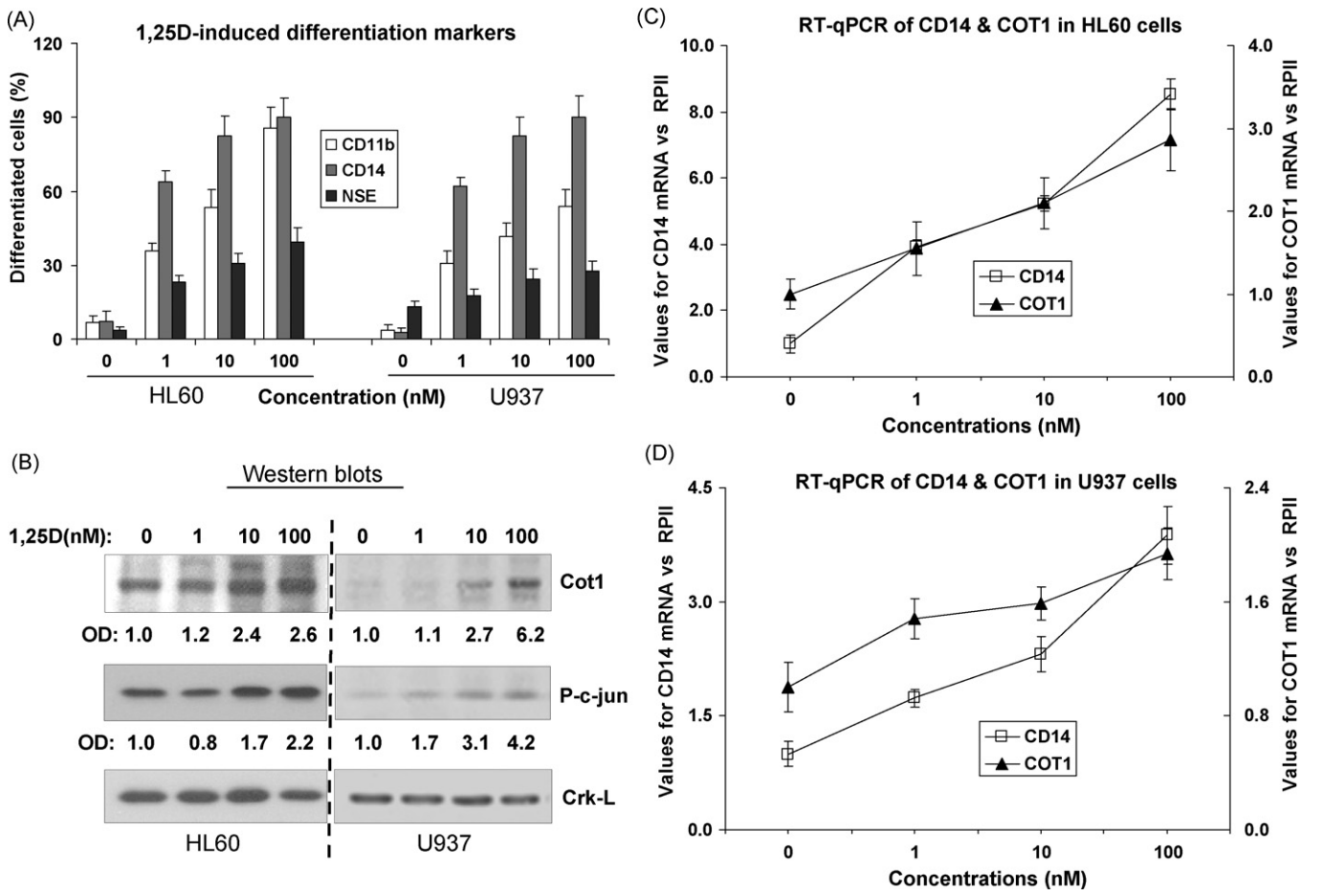

Fig. 1. COT1 expression increases in parallel with 1,25D-induced differentiation. (A) 1,25D concentration-dependent increases in the indicated markers of monocytic phenotype. (B) Western blots showing the 1,25D concentration-dependent increase in COT1 expression, and in a molecular marker of monocytic phenotype, phospho c-jun, Crk-L levels are shown as loading and transfer controls. (C) Comparison of the transcript levels for COT1 and the differentiation marker CD14 in HL60 cells, which show the dose-response relationship in a parallel manner. (D) Same as C, but in U937 cells. In all panels of this figure exposure to 1,25D was 48 h, and mean values ± SD are shown ($n = 3$).

Santa Cruz Biotechnology. The specific pharmacological inhibitor of COT-1 kinase, 4-(3-chloro-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino-3-cyano-[1–7]-naphthyridine (COT Inh) [13–15], was purchased from EMD Chemicals Inc (Gibbstown, NJ).

2.3. Quantitative real-time PCR

Real-time PCR was carried out by using a lightcycler with Faststart DNA SYBR Green PCR kit (Roche Diagnostics, Indianapolis, IN) as previously described [16]. Primers used for real-time PCR were: COT1 upstream 5′-CAAGGCCGCAGATGCAATCTT-3′, downstream 5′-AGTCAGACTCCTGGCTTTGCA-3′; CD14:upstream 5′-AACTCCCTCAATCTGTCGTTCGCT-3′, downstream 5′-GGGCAAAGGGTTGAATTGGTCGAA-3′.

2.4. Western blotting

Western blotting was performed using whole cell extracts according to standard procedures [12].

2.5. Cell cycle analysis

The DNA content of HL60 cells was determined by harvesting one million cells and washing twice with PBS. These cells were then fixed with 75% ethanol at −20 °C for 24 h. Cells were then resuspended in 1 ml of PBS with RNase (at 1 μg/ml, Sigma) and propidium iodide (at 10 μg/ml, Sigma) for 30 min at 37 °C. The PI stained cells were analyzed using an EPICS flow cytometer. The resultant histogram of DNA content was gated and analyzed using the Multicycle program to determine the proportions of cells in each phase of the cell cycle.

2.6. Statistical methods

Each experiment was performed at least three times and the results were expressed as percentages (mean ± S.D.) of the vehicle controls. Significance of the differences between mean values was assessed by a two-tailed Student's *t*-test.

3. Results

3.1. COT1 expression increases during 1,25D-induced monocytic differentiation of human myeloid leukemia cells

As documented in numerous studies [17–19], exposure of human myeloid leukemia cells to 1,25D results in the by-pass of the mutation-induced block to differentiation [20], evidenced by the presence of monocytic markers such as surface proteins CD11b and CD14, as well as by the activity of the cytoplasmic enzyme non-specific esterase (NSE). This is illustrated by an experiment shown in Fig. 1A, where HL60 (FAB subtype M2) and U937 (FAB subtype M4) cells were treated for 48 h with increasing concentrations of

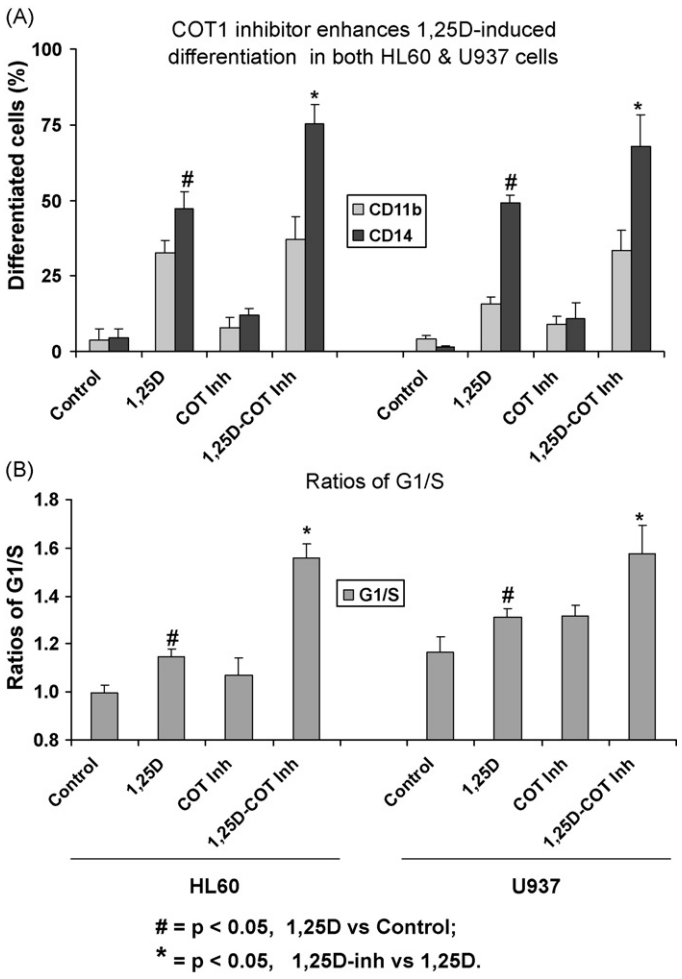

Fig. 2. Inhibition of COT1 kinase activity enhances 1,25D-induced terminal differentiation. (A) Expression of monocytic differentiation markers following 48-h exposure to 1,25D (1 nM), a specific COT1 inhibitor (5 μM), or a combination of both. (B) The ratios of the cell cycle phases G1 to S in cells treated as described in panel A. An increased G1/S ratio indicates cell cycle block. Mean values ± SD are shown ($n = 4$). $^{\#}p < 0.05$, 1,25D vs untreated cells; $^{*}p < 0.05$, 1,25D+ inhibitor vs 1,25D only.

1,25D and the expression of these markers was found to increase in a dose-dependent manner. Interestingly, this increase paralleled the increased expression of COT1 mRNA in both HL60 and U937 cells (Fig. 1C and D). The 1,25D-induced increase in COT1 expression was also demonstrated at protein level as shown by Western blots illustrated in Fig. 1B. Thus, these data suggest that COT1 participate in the events that regulate 1,25D-induced differentiation.

3.2. Activation of c-jun increases in parallel with COT1 expression in 1,25D-treated human myeloid leukemia cells

Previous work in this laboratory has shown that the JNK1/2 pathway, as evidenced by activation of c-jun, contributes to 1,25D-induced differentiation [21,22]. We therefore tested if phospho c-jun (P-c-jun Ser 63) level increases in parallel with COT1, and found that this is the case in both myeloid leukemia cell lines studied here, further establishing a link between COT1 expression and monocytic differentiation of these cells (Fig. 1B).

3.3. Inhibition of kinase activity of COT1 enhances the 1,25D-induced monocytic differentiation

Based on the above results we speculated that COT1 kinase contributes to the events that culminate in monocytic differentiation. Surprisingly, however, we found that pharmacological inhibition of COT1 kinase activity by a specific inhibitor of this enzyme, dubbed "COT Inh", actually potentiates the differentiating action of 1,25D, as shown in Fig. 2A, and this is supported by the increased cell cycle arrest when the 1,25D exposure is supplemented by addition of the COT1 inhibitor (Fig. 2B). These results suggest that COT1 has a negative effect on terminal monocytic differentiation, even though its expression increases while cells differentiate.

4. Discussion

The conclusion reached here that a kinase related to the MAPK pathways increases during differentiation but has a negative effect on this process is dependent on the specificity of the pharmacological inhibitor of COT1 kinase activity, but is highly likely to be valid. The reasons include the documentation that naphthyrine-3-carbonitriles which are COT1 inhibitors have affinity for COT1 which is at least 2000- to 10,000-fold higher than many kinases such as Raf1, MEK, or p38 [13], and since it has the very low homology between COT1 and other kinases is likely to have unique structural features [14]. Although our conclusion will need to be further bolstered by future studies with siRNAs which knockdown COT1 expression, and with constructs which ectopically express COT1, our findings suggest that 1,25D-induced differentiation is a finely balanced process in which negative control plays a role. Perhaps this ensures that the differentiation steps are proceeding in an orderly fashion, not permitting the cell to advance along the differentiation pathway, until all resources for a mature functional monocyte are assembled.

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References

[1] C. Chomienne, P. Ballerini, N. Balitrand, M.T. Daniel, P. Fenaux, S. Castaigne, L. Degos, All-trans retinoic acid in acute promyelocytic leukemias. II. In vitro studies: structure-function relationship, Blood 76 (9) (1990) 1710–1717.
[2] J. Miyoshi, T. Higashi, H. Mukai, T. Ohuchi, T. Kakunaga, Structure and transforming potential of the human cot oncogene encoding a putative protein kinase, Mol. Cell. Biol. 11 (8) (1991) 4088–4096.
[3] C. Patriotis, A. Makris, S.E. Bear, P.N. Tsichlis, Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation, Proc. Natl. Acad. Sci. U.S.A. 90 (6) (1993) 2251–2255.
[4] D. Hagemann, J. Troppmair, U.R. Rapp, Cot protooncoprotein activates the dual specificity kinases MEK-1 and SEK-1 and induces differentiation of PC12 cells, Oncogene 18 (7) (1999) 1391–1400.
[5] S. Das, J. Cho, I. Lambertz, M.A. Kelliher, A.G. Eliopoulos, K. Du, P.N. Tsichlis, Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner, J. Biol. Chem. 280 (25) (2005) 23748–23757.
[6] C.D. Dumitru, J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.H. Lin, C. Patriotis, N.A. Jenkins, N.G. Copeland, G. Kollias, P.N. Tsichlis, TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway, Cell 103 (7) (2000) 1071–1083.
[7] C. Patriotis, A. Makris, J. Chernoff, P.N. Tsichlis, Tpl-2 acts in concert with Ras and Raf-1 to activate mitogen-activated protein kinase, Proc. Natl. Acad. Sci. U.S.A. 91 (21) (1994) 9755–9759.
[8] M. Chiariello, M.J. Marinissen, J.S. Gutkind, Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation, Mol. Cell. Biol. 20 (5) (2000) 1747–1758.
[9] T.T. Wang, L.E. Tavera-Mendoza, D. Laperriere, E. Libby, N.B. MacLeod, Y. Nagai, V. Bourdeau, A. Konstorum, B. Lallemant, R. Zhang, S. Mader, J.H. White, Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D_3 target genes, Mol. Endocrinol. 19 (11) (2005) 2685–2695.
[10] R. Gallagher, S. Collins, J. Trujillo, K. McCredie, M. Ahearn, S. Tsai, R. Metzgar, G. Aulakh, R. Ting, F. Ruscetti, R. Gallo, Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia, Blood 54 (3) (1979) 713–733.

[11] C. Sundstrom, K. Nilsson, Establishment and characterization of a human histiocytic lymphoma cell line (U-937), Int. J. Cancer 17 (5) (1976) 565–577.
[12] X. Wang, J.P. Gardner, A. Kheir, M.R. Uskokovic, G.P. Studzinski, Synergistic induction of HL60 cell differentiation by ketoconazole and 1-desoxy analogues of vitamin D_3, J. Natl. Cancer Inst. 89 (16) (1997) 1199–1206.
[13] L.K. Gavrin, N. Green, Y. Hu, K. Janz, N. Kaila, H.Q. Li, S.Y. Tam, J.R. Thomason, A. Gopalsamy, G. Ciszewski, J.W. Cuozzo, J.P. Hall, S. Hsu, J.B. Telliez, L.L. Lin, Inhibition of Tpl2 kinase and TNF-alpha production with 1,7-naphthyridine-3-carbonitriles: synthesis and structure-activity relationships, Bioorg. Med. Chem. Lett. 15 (23) (2005) 5288–5292.
[14] Y. Hu, N. Green, L.K. Gavrin, K. Janz, N. Kaila, H.Q. Li, J.R. Thomason, J.W. Cuozzo, J.P. Hall, S. Hsu, C. Nickerson-Nutter, J.B. Telliez, L.L. Lin, S. Tam, Inhibition of Tpl2 kinase and TNFalpha production with quinoline-3-carbonitriles for the treatment of rheumatoid arthritis, Bioorg. Med. Chem. Lett. 16 (23) (2006) 6067–6072.
[15] K. Hirata, H. Taki, K. Shinoda, H. Hounoki, T. Miyahara, K. Tobe, H. Ogawa, H. Mori, E. Sugiyama, Inhibition of tumor progression locus 2 protein kinase suppresses receptor activator of nuclear factor-kappaB ligand-induced osteoclastogenesis through down-regulation of the c-Fos and nuclear factor of activated T cells c1 genes, Biol. Pharm. Bull. 33 (1) (2010) 133–137.
[16] X. Wang, T.T. Wang, J.H. White, G.P. Studzinski, Expression of human kinase suppressor of Ras 2 (hKSR-2) gene in HL60 leukemia cells is directly upregulated by 1,25-dihydroxyvitamin D_3 and is required for optimal cell differentiation, Exp Cell Res. 313 (14) (2007) 3034–3045.
[17] E. Abe, C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, T. Suda, Differentiation of mouse myeloid leukemia cells induced by 1 alpha, 25-dihydroxyvitamin D_3, Proc. Natl. Acad. Sci. U.S.A. 78 (8) (1981) 4990–4994.
[18] D.J. Mangelsdorf, H.P. Koeffler, C.A. Donaldson, J.W. Pike, M.R. Haussler, 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells, J. Cell Biol. 98 (2) (1984) 391–398.
[19] G.P. Studzinski, A.K. Bhandal, Z.S. Brelvi, A system for monocytic differentiation of leukemic cells HL 60 by a short exposure to 1,25-dihydroxycholecalciferol, Proc. Soc. Exp. Biol. Med. 179 (3) (1985) 288–295.
[20] G.P. Studzinski, X. Wang, Y. Ji, Q. Wang, Y. Zhang, A. Kutner, J.S. Harrison, The rationale for deltanoids in therapy for myeloid leukemia: role of KSR-MAPK-C/EBP pathway, J. Steroid Biochem. Mol. Biol. 97 (1–2) (2005) 47–55.
[21] Q. Wang, X. Wang, G.P. Studzinski, Jun N-terminal kinase pathway enhances signaling of monocytic differentiation of human leukemia cells induced by 1,25-dihydroxyvitamin D_3, J. Cell. Biochem. 89 (6) (2003) 1087–1101.
[22] X. Chen-Deutsch, E. Garay, J. Zhang, J.S. Harrison, G.P. Studzinski, c-Jun N-terminal kinase 2 (JNK2) antagonizes the signaling of differentiation by JNK1 in human myeloid leukemia cells resistant to vitamin D, Leuk. Res. 33 (10) (2009) 1372–1378.