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Triple combination of retinoic acid plus actinomycin D plus dimethylformamide induces differentiation of human acute myeloid leukaemic blasts in primary culture

Hassan Tawhid Hassan and John Rees

Department of Haematological Medicine, University of Cambridge Clinical School, MRC Building, Cambridge, England, U.K.

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Summary. Differentiation induction therapy provides an alternative for treatment of acute myeloid leukaemia (AML) patients who are either unsuitable for or unresponsive to conventional cytotoxic chemotherapy. The effect of a triple combination of retinoic acid (RA) + actinomycin D (Act-D) + dimethylformamide (DMF) on differentiation of blasts from 24 AML patients was studied. Non-adherent mononuclear cells were seeded at a concentration of 5×10^5 cells/ml in 24-well tissue-culture plates containing RPMI 1640 culture medium with 20% fetal calf serum, 10% autologous serum and 10% 5637-conditioned medium and incubated with 10^{-6} *M* retinoic acid, 5 n*M* actinomycin D and/or 100 m*M* dimethylformamide alone and in combination with each other for 6 days at 37° C in a humidified incubator and an atmosphere containing 5% CO_2. The triple combination of 10^{-6} *M* retinoic acid + 5 n*M* actinomycin D + 100 m*M* dimethylformamide induced 90% of the blasts from 22 of the 24 AML patients to differentiate. The combination of *N*-methylformamide (a compound similar to dimethylformamide) with cyclophosphamide significantly increased the in vivo activity with no concomitant increase in its reversible hepatotoxicity. Since several polar compounds related to dimethylformamide, e.g. hexamethylene bisacetamide and *N*-methylformamide, are currently undergoing phase II clinical trials, it may be feasible to combine one of these with retinoic acid and/or actinomycin D in the treatment of AML patients.

Introduction

Acute myeloid leukaemia (AML) arises from a neoplastic transformation at the stem-cell stage, leading to an arrest of myeloid cell differentiation [6]. The leukaemic cells fail to differentiate, remain in the proliferative pool and rapidly accumulate [23]. Nevertheless, leukaemic cells have not completely lost their potential to differentiate [13]. Human myeloid leukaemic cell lines have been reported to differentiate after exposure to numerous differentiating agents [7]. Differentiation induction therapy has been proposed for AML patients who are either unsuitable for or unresponsive to conventional cytotoxic chemotherapy [8] as well as for patients with myelodysplastic syndromes [11].

At a concentration of 10^{-6} *M*, retinoic acid has been shown to induce the differentiation of 90% of HL-60 human promyelocytic leukaemia cells and 80% of U-937 human monoblastic leukaemia cells [17]. Moreover, 10^{-6} *M* retinoic acid has been reported to induce partial differentiation of blasts from some AML patients in primary culture [1, 9, 16], an effect which was markedly enhanced by its combination with DNA synthesis inhibitors [4, 9]. At a concentration of 5 n*M*, actinomycin D has been reported to induce differentiation of 80% of HL-60 human promyelocytic leukaemia cells, 50% of K562 human erythroleukaemic cells and 45% of ML-1 human myeloblastic leukaemia cells [18]. In addition, 5 n*M* actinomycin D has been shown to stimulate the differentiation of normal human marrow myeloid progenitor cells [10]. At a concentration of 100 m*M*, dimethylformamide has been reported to induce differentiation of 85% of HL-60 human promyelocytic leukaemic cells [2] and to stimulate the differentiation of immature, normal human marrow myeloid cells [12].

Since the heterogeneity of leukaemic cells can limit the effectiveness of a single differentiating agent, it is of clinical importance to seek combinations of more than one differentiating agent that can act on different portions of the leukaemic population to induce maximal differentiation [9]. In the present study, the response of AML blasts to 10^{-6} *M* retinoic acid, 5 n*M* actinomycin D and/or 100 m*M* dimethylformamide alone and in combination with each other in primary culture was examined. The results show that the triple combination of 10^{-6} *M* retinoic acid + 5 n*M* actinomycin D + 100 m*M* dimethylformamide induced differentiation of 90% of the blasts from 22 of 24 AML patients on day 6 of primary culture.

Patients and methods

Cell preparation. Samples of 5–10 ml heparinised peripheral blood were obtained from each of 24 AML patients [6 AMyL (M1), 6 AML (M2), 3 APL (M3), 5 AMMoL (M4) and 4 AMoL (M5)] at the time of diagnosis before therapy. Only specimens containing >70% blasts were studied. Mononuclear cells were isolated by layering over Ficoll-Hypaque (density, 1.007) and centrifugation for 25 min at 600 *g*. Non-adherent mono-

Offprint requests to: H. T. Hassan, Darwin College, Silver Street, Cambridge CB3 9EU, England, U.K.

Table 1. Effect of 10^{-6} *M* retinoic acid, 5 n*M* actinomycin D and/or 100 m*M* dimethylformamide alone and in combination with each other in primary culture on AML cell survival

Differentiating Agent	Concentration	Viable cells (*n*)[a] ($\times 10^5$/ml)
Control	–	8.9±2.8
Retinoic acid (RA)	10^{-6} *M*	7.2±1.6
Actinomycin D (Act-D)	5 n*M*	6.3±1.2
Dimethylformamide (DMF)	100 m*M*	6.4±2.5
RA + Act-D	10^{-6} *M* + 5 n*M*	5.8±1.9
RA + DMF	10^{-6} *M* + 100 m*M*	6.1±1.7
Act-D + DMF	5 n*M* + 100 m*M*	5.5±2.7
RA + Act-D + DMF	10^{-6} *M* + 5 n*M* + 100 m*M*	5.3±2.3

[a] The initial viable cell count on day 0 was 5×10^5 cells/ml

nuclear cells were obtained by two cycles of incubation in plastic tissue-culture plates, each for 1 h at 37° C in a humidified incubator and an atmosphere containing 5% CO_2. The viability of cells was >96% as determined by the trypan blue exclusion test. These cells were composed of >94% blast cells as judged by morphological examination of Hyel-Romanowsky-stained cytocentrifuge smears on day 0.

Cell culture. Non-adherent mononuclear cells were cultured at a concentration of 5×10^5 cells/ml in 24-well tissue-culture plates containing RPMI 1640 culture medium with 20% fetal calf serum, 10% autologous serum and 10% 5637-conditioned medium, which was prepared as previously described [15]. Cells were then incubated with 10^{-6} *M* retinoic acid, 5 n*M* actinomycin D and 100 m*M* dimethylformamide alone and in combination with each other for 6 days at 37° C in a humidified incubator and an atmosphere containing 5% CO_2. A control culture containing neither retinoic acid, actinomycin D nor dimethylformamide was also set up under otherwise identical conditions.

Assessment of differentiation on day 6. Using Hyel-Romanowsky stain, we morphologically prepared the cytocentrifuge smears on day 6 of primary culture; at least 200 cells were scored for morphological maturation. The dual esterase staining of cytocentrifuge smears on day 6 was carried out as previously described [27]; at least 200 cells were scored for both chloroacetate esterase- and butyrate esterase-positive cells. The nitroblue tetrazolium dye-reducing test was carried out as previously described [9], and at least 200 cells were scored for the presence of intracellular blue-black formazan deposits.

Results

After 6 days of primary culture, the viability of cells in both control and treated cultures was >74%. Incubation for the entire 6 days with 10^{-6} *M* retinoic acid, 5 n*M* actinomycin D and 100 m*M* dimethylformamide alone and in combination with each other did not reduce their growth by >40%. Spontaneous differentiation after 6 days of primary culture was found in control cultures of only 3 (1 M2, 1 M3, 1 M4) of the 24 AML patients studied. Evidence for the leukaemic origin of these differentiated cells included the correlation of specific types of mature cells appearing in primary treated cultures on day 6 with the FAB subtype of their AML blasts and the appearance of Auer rods in the differentiated cells of treated cultures in one case (Table 1).

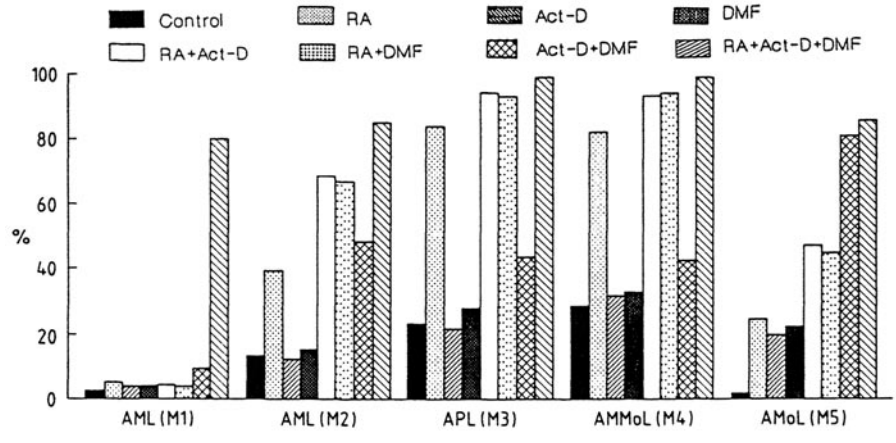

Fig. 1. FAB subtypes of acute myeloid leukaemia. Response of AML blasts to 1 μ*M* retinoic acid (*RA*), 5 n*M* actinomycin D (*Act-D*) and/or 100 m*M* dimethylformamide (*DMF*) alone and in combination in primary culture. *Morphologically mature cells* include mature granulocytes and monocytes

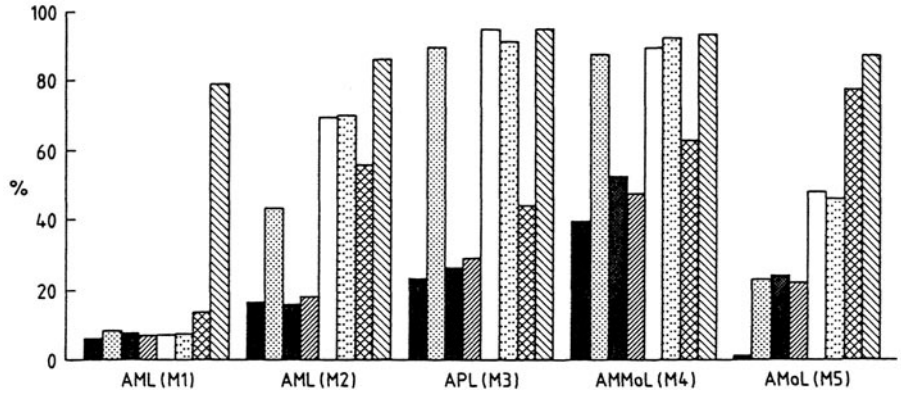

Fig. 2. FAB subtypes of acute myeloid leukaemia. Response of AML blasts to 1 μ*M* RA, 5 n*M* Act-D and/or 100 μ*M* DMF alone and in combination in primary culture (esterases + ve cells). Symbols are the same as those shown in Fig. 1

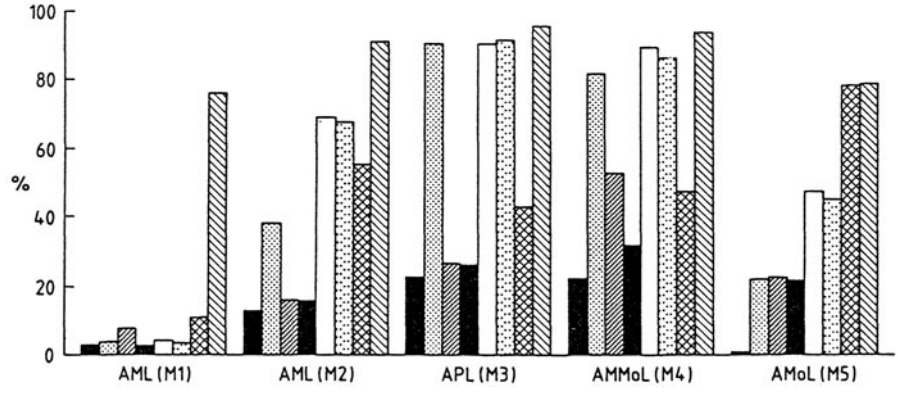

Fig. 3. FAB subtypes of acute myeloid leukaemia. Response of AML blasts to 1 μ*M* RA, 5 n*M* Act-D and/or 100 μ*M* DMF alone and in combination in primary culture (nitroblue tetrazolium dye-reducing cells). Symbols are the same as those shown in Fig. 1

Table 2. Induction of differentiation of human AML blasts in primary culture (n = 24 patients)

Differentiating agent	Morphologically mature cells (%) [a]	*P**	Esterases + ve cells (%) [b]	*P*	Nitroblue tetrazolium dye-reducing cells (%)	*P*
Control (none)	13.2±11.5	–	16.2±15.6	–	11.4± 9.3	–
Retinoic acid (RA) 10^{-6} *M*	43.7±32.6	<0.001	48.8±12.7	<0.001	43.6±34.5	<0.001
Actinomycin D (Act-D) 5 n*M*	18.3±12.0	NS	23.8±13.2	NS	18.7±11.6	NS
Dimethylformamide (DMF) 100 m*M*	19.5±11.6	NS	18.9±11.8	NS	18.1±10.9	NS
RA + Act-D	51.1±32.9	<0.001	53.4±37.2	<0.001	49.6±31.7	<0.001
RA + DMF	50.6±32.8	<0.001	53.5±37.1	<0.001	46.3±32.0	<0.001
Act-D + DMF	42.2±24.9	<0.001	48.6±29.7	<0.001	33.6±23.7	<0.001
RA + Act-D + DMF	89.6± 7.5	<0.0001	89.9± 6.1	<0.0001	84.8± 4.9	<0.0001

[a] Including metamyelocytes, mature granulocytes and monocytes
[b] Including chloroacetate and butyrate esterases + ve cells
* *t*-Distribution test in comparisons with controls; NS, not significant

After 6 days of primary culture, 10^{-6} *M* retinoic acid increased the number of morphologically mature cells, esterase + ve cells and nitroblue tetrazolium dye-reducing cells from only 13.2%±11.5%, 16.2%±15.6% and 11.4%±9.3% in control cultures to 43.7%±32.6%, 48.8%±12.7% and 43.1%±34.5%, respectively (Fig. 1–3). The response of AML blasts to 10^{-6} *M* retinoic acid in primary culture varied among the 24 patients studied. Whereas most AML blasts from patients with acute promyelocytic leukaemia (APL)M3 and acute myelomonocytic leukaemia (AMMoL)M4 were induced to differentiate after 6 days of primary culture with 10^{-6} *M* retinoic acid, those from patients with acute myeloblastic leukaemia (AML)M2 and acute monoblastic leukaemia (AMoL)M5 showed only partial maturation and those from patients with acute myeloblastic leukaemia (AML)M1 were resistant (Fig. 1–3).

Both 5 n*M* actinomycin D and 100 m*M* dimethylformamide were weak inducers of differentiation of human AML blasts in primary culture (Figs. 1–3). The blasts

from patients with AMoL(M5) were the only FAB subtype to respond to these two agents with partial differentiation, an effect that was enhanced by their combination only in this particular subtype (Figs. 1–3). Whereas the addition of either 5 n*M* actinomycin D or 100 m*M* dimethylformamide to retinoic acid-treated cultures containing blasts from patients with AML(M2) and AMoL(M5) increased the number of morphologically mature cells, esterase + ve cells and nitroblue tetrazolium dye-reducing cells to 63.1%±9.9%, 67.1%±12.0% and 61.3%±12.5% and 44.0%±3.5%, 47.9%±5.1% and 37.0%±2.8%, respectively, the blasts from patients with AML(M1) remained resistant (Figs. 1–3). Not only did the triple combination of 10^{-6} *M* retinoic acid + 5 n*M* actinomycin D + 100 m*M* dimethylformamide induce maximal differentiation of >85% of the blasts from all patients with AML(M2), APL(M3), AMMoL(M4) and AMoL(M5), but it also more significantly increased the number of morphologically mature cells, esterase + ve cells and nitroblue tetrazolium dye-reducing cells in cultures containing blasts from four of the six AML(M1) patients to 90.1%±6.8%, 93.2%±4.1% and 84.0%±6.7%, respectively (Figs. 1–3).

Table 2 shows a statistically significant difference in the number of morphologically mature cells, esterases + ve cells and nitroblue tetrazolium dye-reducing cells between control and retinoic acid-treated cultures on day 6. This statistically significant difference did not increase after the addition of 5 n*M* actinomycin D or 100 m*M* dimethylformamide alone to retinoic acid-treated cultures but was profoundly increased by the addition of both 5 n*M* actinomycin D and 100 m*M* dimethylformamide (Table 2).

Discussion

The major finding of the present study is that the triple combination of retinoic acid + actinomycin D + dimethylformamide induced differentiation in >90% of blasts from 22 of the 24 AML patients studied after 6 days of primary culture. The exact mechanism of action of these differentiating agents is still unknown. One explanation is that such agents act on different stages of the differentiation process and can therefore produce by complementation the stimulus required for maximal differentiation.

Some reports recently suggested a two-step model for inducing terminal differentiation by which the process of myeloid cell differentiation can be resolved into early events leading to precommitment and late events leading from precommitment to commitment and cell maturation [26, 28]. Whereas early events anteceding precommitment regulate growth arrest, late events subsequent to precommitment regulate the choice of a specific differentiation lineage [29]. The results of several preliminary reports have shown an excellent response to retinoic acid given alone only in APL patients [25]. This drug showed a striking advantage over conventional cytotoxic chemotherapy for APL patients; instead of destroying leukaemic cells and causing the release of procoagulant factors from the azurophilic granules into the circulation, which usually results in disseminated intravascular coagulopathy (DIC), retinoic acid induced leukaemic cell differentiation in APL patients and thus avoided the release of such coagulant factors during therapy [25].

Francis et al. [5] recently reported that seven AML and MDS patients who failed to respond to single-agent differentiation therapy showed a clinical response after treatment with retinoic acid + DNA synthesis inhibitor, suggesting that such a combination may be more effective than single-agent differentiation therapy. Since the combination of *N*-methylformamide (a compound similar to dimethylformamide) with cyclophosphamide significantly increased the in vivo activity with no concomitant increase in its reversible hepatotoxicity [21], it may be feasible to combine polar compounds related to dimethylformamide, e.g. hexamethylene bisacetamide and *N*-methylformamide, with retinoic acid and/or actinomycin D in phase II clinical trials in AML patients.

High-dose retinoic acid has been given for up to 4 years in AML patients in remission, with no significant clinical or biochemical toxic effects [22]; this was believed to involve a metabolic adaptation through increasing the biliary excretion of retinol metabolites as the liver retinol concentration increases [14, 20]. Since the treatment of myeloid leukaemic cells with a combination of differentiation inducers and low-dose antileukaemic drugs has been reported to suppress the emergence of differentiation-resistant cells [19], the triple combination of retinoic acid + actinomycin D + dimethylformamide may be beneficial as an effective maintainance therapy for AML patients in remission.

References

1. Brietman TR, Collins SJ, Kenne BR (1981) Terminal differentiation of human promyelocytic leukaemia cells in primary culture in response to retinoic acid. Blood 57: 1000
2. Collins SJ, Bodner A, Ting R, Gallo RC (1980) Induction of both morphological and functional differentiation of human HL-60 promyelocytic leukaemic cells by compounds which induce differentiation of murine leukaemic cells. Int J Cancer 25: 213
3. Ettinger DS, Orr DW, Rice AP, Donehower RC (1985) Phase I study of *N*-methylformamide in patients with advanced cancer. Cancer Treat Rep 69: 489
4. Francis GE, Guimaraes JETE, Berney JJ, Wing MA (1985) Synergistic interaction between differentiation inducers and DNA synthesis inhibitors: a new approach to differentiation induction in myelodysplasia and acute myeloid leukaemia. Leuk Res 9: 573
5. Francis GE, Mufti GJ, Knowles SM, Berney JJ, Guimaraes JETE, Secker-Walker LM, Hamblin TJ (1987) Differentiation induction in myelodysplasia and acute myeloid leukaemia: use of synergistic drug combinations. Leuk Res 11: 971
6. Hassan HT (1988) Differentiation induction therapy of acute myelogenous leukaemia. Haematologia 21: 141
7. Hassan HT (1988) Differentiation inducers of human myeloid leukaemic cell lines. Folia Haematol Leipz 115: 887
8. Hassan HT (1988) Differentiation induction therapy: an alternative for the treatment of elderly patients with acute myeloid leukaemia. J Clin Exp Gerontol 10: 63
9. Hassan HT, Rees JKH (1988) Retinoic acid alone and in combination with cytosine arabinoside induces differentiation of human myelomonocytic and monoblastic leukaemia cells. Haematol Oncol 6: 39
10. Hassan HT, Rees JKH (1989) Low concentrations of cytosine arabinoside, 6-thioguanine, actinomycin D and aclacinomycin A stimulates the differentiation of human normal marrow myeloid progenitor cells. Med Oncol Tumor Pharmacother 6: 213
11. Hassan HT, Rees JKH (1989) Differentiation induction therapy of myelodysplastic syndromes. Leuk Res 13: 633

12. Hassan HT, Rees JKH (1989) Effect of polar-planar compounds on the differentiation of normal human myeloid immature marrow cells. Cancer Lett 46: 37
13. Hassan HT, Labastide W, Barker C, Rees JKH (1989) Cooperative effects of human recombinant GM-CSF and human recombinant erythropoietin in inducing the erythroid differentiation of human erythroleukaemic cell line K562 clonogenic cells. Leuk Res 13: 127
14. Hicks V, Gunning D, Olson J (1984) Metabolism, plasma transport and biliary excretion of radioactive vitamin A and its metabolites as a function of liver reserves of vitamin A. Am J Nutr 114: 1327
15. Hoang T, McCulloch EA (1985) Production of leukaemic blast growth factor by a human bladder carcinoma cell line. Blood 66: 748
16. Honma Y, Fujita Y, Hozumi M, Sampi K, Sakuri M, Tshusuima A, Hiroski N (1983) Induction of differentiation of human acute nonlymphocytic leukaemia cells in primary culture by inducers of differentiation of human myeloid leukaemic cell line HL-60. Eur J Cancer Clin Oncol 19: 251
17. Hozumi M (1982) Differentiation inducers of human myeloid leukaemia cells and anticancer agents. Nippon Rinsho 40: 1865
18. Hozumi M (1983) Fundamentals of chemotherapy of myeloid leukaemia cell differentiation. Adv Cancer Res 38: 121
19. Kasukabe T, Honma Y, Hozumi M, Suda T, Nishii Y (1987) Control of proliferating potential of myeloid leukaemia cells during long term treatment with vitamin D analogues and other differentiation inducers in combination with antileukaemic drugs: in vitro and in vivo studies. Cancer Res 47: 567
20. Korner W, Vollm L (1975) New aspects of the tolerance of retinols in humans. Int J Vit Nutr Res 45: 363
21. Langdon SP, Hickman JA, Gescher A, Stevens HFG, Chubb D, Vickers LM (1985) *N*-Methylformamide (NSC 3051): a potential candidate for combination chemotherapy. Eur J Cancer Clin Oncol 21: 745
22. Lie SO, Wathne K, Peterson LB, Slordehl SH, Norum KR (1988) High dose retinol in children with AML in remission. Eur J Haematol 40: 460
23. Lubbert M, Koeffler HP (1988) Myeloid cell lines: tools for studying the differentiation of normal and abnormal haemopoietic cells. Blood Rev 2: 121
24. McVie JG, Ten Bokkel HWW, Simonetti G (1984) Phase I clinical trial of *N*-methylformamide. Cancer Treat Rep 68: 607
25. Meng-er H, Yu-Chen Y, Shu-rong C, Jin-ren C, Jia-Xiang L, Lin Z, Long-jun G, Zhen-yi W (1988) Use of all-trans retinoic acid in the treatment of acute promyelocytic leukaemia. Blood 72: 567
26. Sachs L (1987) The molecular regulators of normal and leukaemic blood cells. Proc R Soc Lond [Biol] 231: 289
27. Swirsky DM (1984) Single incubation double esterase cytochemical reaction using a single coupling reagent. J Clin Pathol 37: 1187
28. Yen A, Albright K (1984) Evidence of cell cycle phase specific initiation of a program of HL-60 cell myeloid differentiation mediated by inducer uptake. Cancer Res 44: 2511
29. Yen A, Forbes M, DeGala G, Fishbaugh J (1987) Control of HL-60 cell maturation lineage specificity, a late event occurring after the precommitment. Cancer Res 47: 129