Pergamon

J Steroid Biochem Molec. Biol Vol. 48, No 1, pp. 95–99, 1994

0960-0760/94 $6.00 + 0 00

Neopterin Release by Myeloid Leukaemic Cells can be Synergistically Augmented by 1,25-Dihydroxyvitamin D3 in Combination with Gamma Interferon or Granulocyte-macrophage Colony Stimulating Factor

S. M. Kelsey,[1]* R. D. Coldwell,[2] H. L. J. Makin,[2] D. Syndercombe-Court[1] and A. C. Newland[1]

Departments of [1]Haematology and [2]Chemical Pathology, The Royal London Hospital, Whitechapel, London E1 1BB, England

Neopterin is a pteridine molecule released by immune activated monocytes. Monocytic maturation may be induced in acute myeloid leukaemia (AML) blasts and the U937 leukaemic cell line by 1,25-dihydroxyvitamin D3 [1,25(OH)$_2$D3], an effect which is augmented by both gamma interferon (IFN) or granulocyte-macrophage colony stimulating factor (GM-CSF). We have demonstrated that, while 1,25(OH)$_2$D3 and GM-CSF alone have little effect, both IFN and GM-CSF act synergistically with 1,25(OH)$_2$D3 to increase neopterin secretion in the U937 cell line. Neopterin secretion was associated with, but not necessarily dependent on, the degree of phenotypic differentiation achieved by cells. Neopterin secretion was also synergistically enhanced in AML blasts by the action of 1,25(OH)$_2$D3 in combination with IFN but not GM-CSF; secretion was enhanced in AML blasts without concomitant evidence of phenotypic maturation. We have shown that the monocytoid cell line U937, under appropriate conditions, may secrete neopterin in response to stimulatory agents other than IFN. In addition, the distinct difference in the pattern of response to the combination of 1,25(OH)$_2$D3 with GM-CSF compared with that of 1,25(OH)$_2$D3 plus IFN suggests that the augmentation of 1,25(OH)$_2$D3 effect by IFN and GM-CSF is mediated by separate mechanisms.

J. Steroid Biochem. Molec. Biol., Vol. 48, No. 1, pp. 95–99, 1994

INTRODUCTION

Neopterin is a pteridine compound derived from guanosine triphosphate (GTP) which is secreted by activated monocytes [1, 2]. Two forms exist in humans; 7,8 dihydroneopterin triphosphate which is active intracellularly and 7,8-dihydroneopterin which is predominant in body fluids [3].

Raised neopterin levels have been found *in vivo* during clinical states of immune activation, such as acquired immune deficiency syndrome (AIDS) [4, 5], rheumatoid arthritis [6, 7], graft versus host disease (GvHD) [8] and during regeneration following bone marrow transplantation [9]. *In vitro* studies have shown that monocytes will release neopterin when stimulated directly with gamma interferon (IFN) or bacterial lipopolysaccharide (LPS) but not by other cytokines which activate other aspects of monocyte function such as granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 1 (IL1) or tumor necrosis factor (TNF) [10, 11].

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 [1,25(OH)$_2$D3] will induce monocytic differentiation of marrow precursors [12] and activation of mature monocytes [13]. Monocytic maturation can also be induced by 1,25(OH)$_2$D3 in human myeloid leukaemic blasts and some leukaemic cell lines, such as the monoblastic cell line U937 [14–16]. 1,25(OH)$_2$D3-induced differentiation can be synergistically augmented by IFN or GM-CSF [17, 18]. We therefore studied the effect on neopterin secretion by U937 cells of the induction of differentiation by 1,25(OH)$_2$D3, IFN and GM-CSF, alone and in combination. We also studied the effect of these agents on neopterin secretion by blasts from patients with acute myeloid

*Correspondence to S M. Kelsey
Received 29 March 1993, accepted 16 Sep. 1993

leukaemia (AML), which varied in their susceptibility to differentiation–induction.

EXPERIMENTAL

Suspension culture

Fresh leukaemic blasts were isolated from the peripheral blood of patients with AML by gradient centrifugation over Lymphoprep 1.077 (Nycomed, Oslo). Adherent cells were depleted by plating in 80 ml plastic flasks at 37°C for 1 h. The presence of pure (>95%) blast populations was confirmed by May Grunwald Giemsa staining of cytospin preparations (Shandon Southern).

Cells of the monoblastoid human leukaemic cell line U937 (seeded at 0.3–0.6 × 10^6 cells/ml) and purified AML blasts (seeded at a mean of 2 × 10^6 cells/ml) were cultured in RPMI 1640 with 10% fetal calf serum (GIBCO, Paisley, Scotland) plus penicillin and streptomycin at 37°C for up to 96 h. Medium was supplemented with $1,25(OH)_2D3$ 1^{-8} M (Roche Products Ltd, England), human gamma IFN 250 U/ml (Sigma Products), recombinant human GM-CSF 5 ng/ml (a kind gift from Dr M. Garnick, Genetics Institute, Cambridge, MA) or a combination of these agents. Stock $1,25(OH)_2D3$ in ethanol was used leading to a final concentration of ethanol in cultures of 0.05%. Control cultures were incubated with a similar concentration of ethanol alone which has been previously demonstrated to have negligible effect on cell function. Medium from U937 cell cultures was removed daily for analysis, being replaced with appropriately supplemented fresh medium. Medium and cells from AML blast cultures were assessed at 72 h. Immediately prior to analysis cell viability was assessed by counting after a 1 min incubation with 0.2% trypan blue.

Monocytic differentiation was assessed by quantifying expression of the monocyte-associated antigen CD14, which has been found in previous studies to correlate with other phenotypic markers of monocytic maturation both in the U937 cell line and AML blasts [17, 19]. Cells were harvested from culture, washed twice in RPMI, and incubated with the fluorescein isothionate (FITC)-conjugated monoclonal antibody anti-LeuM3 (Becton Dickinson). Expression was quantified by flow cytometry (Epics C, Coulter Products) after excitation at 488 nm; background fluorescence was assessed by binding of a non-specific murine anti-IgG1 FITC-conjugated antibody. Adherent cells were included in the analysis following removal with a rubber cell scraper after incubation for 5 min under 2.7% cold EDTA (4°C, pH 7.4). For AML blast populations an increase of 10% or greater in the proportion of blasts showing CD14 positively was deemed to be significant.

Measurement of neopterin levels

Supernatant neopterin levels were measured by radioimmunoassay (Henning Berlin GmbH). Samples (50 μl) were assayed directly by incubation with ^{125}I-labelled neopterin and an antibody to neopterin raised in sheep. Separation of free and bound antibody was achieved using PEG. This assay gave excellent between-batch reproducibility (CV < 5%) over the whole range of concentrations encountered.

Statistical methods

Neopterin secretion by AML blast populations at a single time point were compared by paired Students t-test. Serial neopterin secretion over 96 h by U937 cells was analysed using MANOVA with three repeated measures.

RESULTS

Effect of D3, IFN and GM-CSF on monocytic maturation of U937 cells

This study confirms previous findings that $1,25(OH)_2D3$ induces time-dependent monocytic differentiation of U937 cells (Fig. 1). While both IFN and GM-CSF have little effect alone both agents augment, $1,25(OH)_2D3$-induced maturation in a synergistic manner. In addition GM-CSF, unlike IFN, markedly accelerates the $1,25(OH)_2D3$-induced acquisition of a monocytoid phenotype.

Neopterin secretion by U937 cells

Figure 2 (a and b) show that basal neopterin secretion by U937 cells in culture was consistently low and was not influenced by GM-CSF alone. $1,25(OH)_2D3$ alone exerted a small but significant time dependent increase in one experiment but this was not reproducible. By contrast, IFN alone had a marked effect ($P = 0.003$), neopterin secretion increasing almost 2-fold to reach a maximum after 72 h. Both IFN ($P = 0.03$) and GM-CSF ($P = 0.02$) acted synergistically with $1,25(OH)_2D3$ to increase

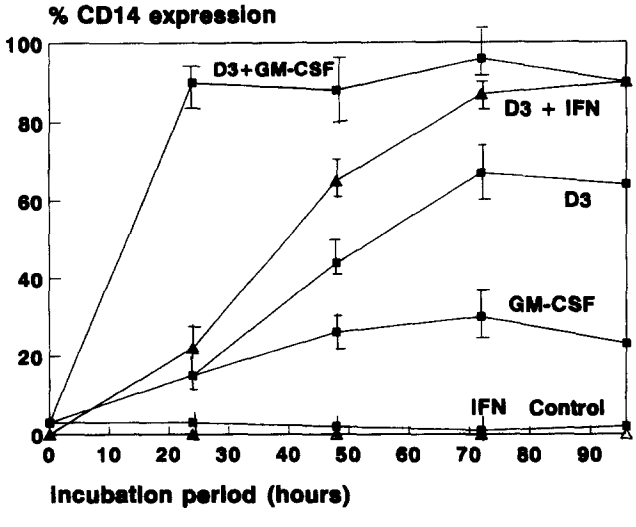

Fig. 1. Acquisition of CD14 antigen by U937 cells over 96 h on stimulation with $1,25(OH)_2D3$ and synergistic augmentation of $1,25(OH)_2D3$-induced differentiation by both IFN and GM-CSF. Figures represent the mean ± standard deviation of 5 independent experiments.

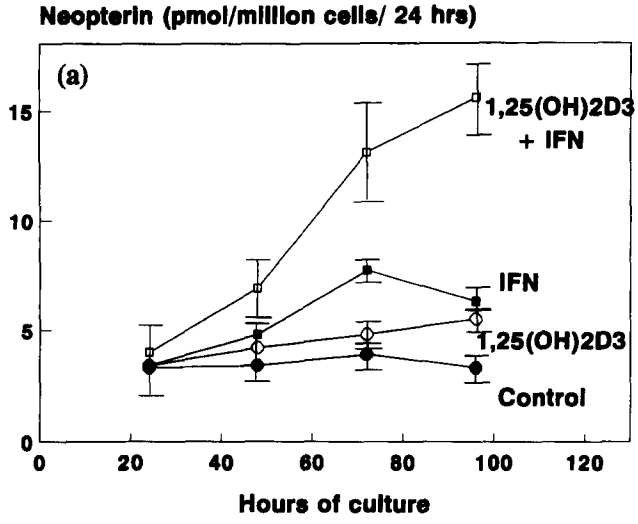

Fig. 2. Increase in neopterin secretion, measured as pmol/million cells/24 h, by U937 cells over 96 h incubation with $1,25(OH)_2D3$ and IFN (a) or GM-CSF (b). Figures represent the mean ± standard deviation of 5 independent experiments. Control, ●—●; $1,25(OH)_2D3$, ○—○; IFN or GM-CSF, ■—■; $1,25(OH)_2D3$ plus either GM-CSF or IFN, □—□.

neopterin secretion. Maximal secretion in the presence of $1,25(OH)_2D3$ and IFN was achieved after 96 h (the last time point of the experiment) whereas the combination of $1,25(OH)_2D3$ plus GM-CSF induced maximal secretion after 48 h of culture.

Optimum ability to secrete neopterin was temporally related to maximal phenotypic differentiation as assessed by CD14 antigen expression. In support of this observation there was a strong correlation between CD14 antigen positivity and neopterin secretion. This was particularly true following stimulation by $1,25(OH)_2D3$, either with or without IFN ($r = 0.67$; $P < 0.001$) or GM-CSF ($r = 0.71$; $P < 0.001$).

Neopterin secretion by AML blasts

In order to assess whether the combined effect of $1,25(OH)_2D3$ plus IFN or GM-CSF was due entirely to induction of monocytic maturation we studied

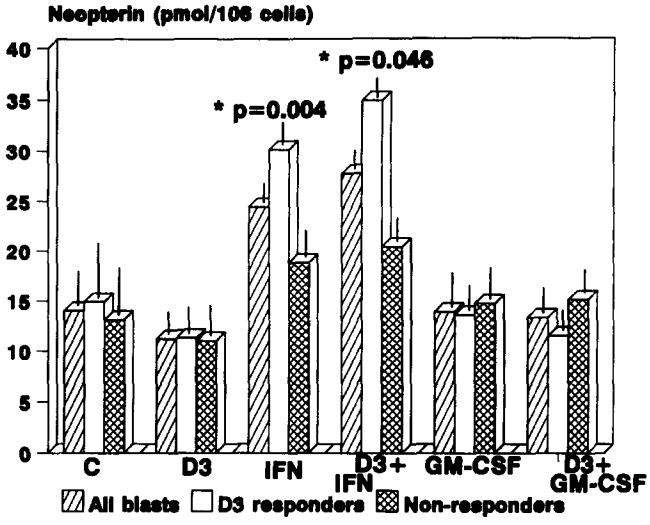

Fig. 3. Mean (±standard deviation) neopterin secretion (pmol/million cells) over 96 h by 10 AML blast populations stimulated with differentiation-inducing agents, showing a small but statistically significant synergistic action between $1,25(OH)_2D3$ and IFN but not GM-CSF. Neopterin secretion by AML blasts which underwent $1,25(OH)_2D3$-induced monocytic maturation ($n = 5$) and AML blasts which were resistant to the differentiation-inducing properties of $1,25(OH)_2D3$ either with or without IFN ($n = 5$) is also shown.

the effect of these agents on neopterin secretion by 10 AML blast populations. Five blast populations showed significant phenotypic differentiation after stimulation with $1,25(OH)_2D3$ and 5 exhibited resistance to $1,25(OH)_2D3$. Mean blast count in control cultures was 90 ± 6% of pre-culture level. Blast viability after 96 h was not significantly different in any treatment group from control cultures.

Unlike their action on U937 cells, $1,25(OH)_2D3$ and GM-CSF, either alone or in combination, had no effect on neopterin secretion by AML blasts (Fig. 3). By contrast IFN increased secretion by nearly 2-fold ($P = 0.004$). In addition, a further small increase in neopterin secretion was observed due to the synergistic action of IFN in combination with $1,25(OH)_2D3$ ($P = 0.046$; Fig. 3). IFN-induced secretion was strongly correlated with monocytic phenotype both before ($r = 0.69$; $P = 0.026$) and following ($r = 0.73$; $P < 0.001$) exposure to $1,25(OH)_2D3$. Those blasts exhibiting monocytic maturation with $1,25(OH)_2D3$ secreted more neopterin on exposure to IFN than those not undergoing differentiation ($P = 0.05$, Mann U Whitney). The combination of $1,25(OH)_2D3$ plus IFN had a small but statistically significant synergistic effect ($P = 0.04$) on neopterin secretion in the 5 blast populations where no phenotypic evidence of differentiation was seen although either agent alone had no effect in this sub-population.

DISCUSSION

Unstimulated U937 cells, and some AML blast populations, consistently secreted low levels of neopterin over a 4 day period of culture. IFN significantly

increased neopterin secretion whereas GM-CSF had no effect; the effect with $1,25(OH)_2D3$ was small inconsistent and time-dependent. In this respect the behaviour of U937 cells and AML blasts was similar to that of human monocytes [10]. Interestingly, $1,25(OH)_2D3$ acted synergistically with both GM-CSF and IFN to increase neopterin secretion, as well as CD14 expression, in the U937 cell line. A small but statistically significant synergistic effect of $1,25(OH)_2D3$ and IFN, but not GM-CSF, was also observed in AML blasts. IFN-induced secretion of neopterin by the THP-1 cell line has been shown to be enhanced by both LPS and TNF alpha [20] and IL2 will increase IFN-mediated neopterin release by the transformed T cell line MT2 [21]. In either instance it is unclear whether the mechanism of interaction is at the transcriptional or post-transcriptional level. $1,25(OH)_2D3$, however, has not previously been shown to modulate neopterin secretion either alone or in combination with other agents.

Alpha IFN, in concentrations some 1000 times greater than gamma IFN, is the only other cytokine which has been demonstrated to induce neopterin release by monocytes [11]. In this respect the synergistic action of GM-CSF and $1,25(OH)_2D3$ is unprecedented. Although trace quantities of IFN in the culture system acting on a phenotypically mature cell may be responsible a direct effect of these two agents interacting via a non-IFN-dependent mechanism cannot be excluded. The observations that GM-CSF and $1,25(OH)_2D3$ synergize to facilitate or increase other functional aspects of monocytic maturation in the U937 cell line such as respiratory burst activity [18] and TNF secretion [22] provide a good precedent for up-regulation of neopterin secretion.

The effect of $1,25(OH)_2D3$, in combination with either IFN or GM-CSF, on neopterin secretion appears to be both time-dependent and strongly linked to the degree of monocytic differentiation induced by these agents. Maturation may occur alongside, and independently of, the increased facility for neopterin release; however, the parallel expression of CD14 antigen and other characteristics of maturation suggest that it is more likely that differentiation down the monocyte pathway is a pre-requisite for significant levels of neopterin secretion. AML blasts, however, showing no evidence of maturation on exposure to either $1,25(OH)_2D3$, with or without IFN, exhibited a synergistic increase in neopterin secretion following culture with these agents. This implies that $1,25(OH)_2D3$ and IFN in combination have a direct effect on neopterin secretion which is, at least in part, independent of phenotypic maturation.

Optimal neopterin secretion by U937 cells occurs earlier following stimulation with $1,25(OH)_2D3$ plus GM-CSF than with $1,25(OH)_2D3$ plus IFN and has a close temporal relationship with the appearance of the CD14 antigen. Neopterin secretion may therefore be used as a marker of monocytic maturation by this cell line. The mechanism of interaction between $1,25(OH)_2D3$ and IFN or GM-CSF is unclear. However, the markedly different patterns of response seen for the two combinations suggests that they are acting via different pathways. This is further reinforced by the failure of $1,25(OH)_2D3$ in combination with GM-CSF to influence neopterin secretion in AML blasts which however did respond to $1,25(OH)_2D3$ and IFN. It is possible that GM-CSF and $1,25(OH)_2D3$ in combination fail to induce maturation of AML blasts to a sufficient degree for neopterin secretion to be significantly affected. Alternatively, the ability of $1,25(OH)_2D3$ plus GM-CSF to induce significant levels of neopterin synthesis may be unique to the U937 cell line and may depend on activation of a signalling mechanism not present in other malignant monocytoid cells. Further studies to examine the modulation of neopterin secretion in normal and neoplastic monocytic cells are required.

REFERENCES

1. Kaufman S., Berlow J. R., Summer G. K., Milstein S , Schulman J. D , Orloff S., Spielberg S. and Pueschel S.: Hyperphenylalaninemia due to a deficiency of bioterin, a variant form of phenylketonuria. *New Engl J Med* **299** (1978) 673–679.
2. Huber C., Batchelor J R., Fuchs D., Hausen A., Lang A., Niederwieser D., Reibnegger G, Swetly P., Troppmair J and Wachter H.: Immune response-associated production of neopterin. release from macrophages primarily under control of interferon gamma. *J Exp. Med.* **160** (1984) 310–316.
3. Editorial. Neopterins in clinical medicine. *Lancet* i (1988) 509–511.
4. Fuchs D., Hausen A , Reibnegger G , Werner E. R., Dierich M. P. and Wachter H.. Neopterin as a marker for activated cell-mediated immunity. *Immun. Today* **9** (1988) 150–155
5. Wachter H., Fuchs D , Hausen A., Reibnegger G., Werner E R. and Dierich M P Who will get AIDS? *Lancet* ii (1986) 1216–1217
6. Reibnegger G., Egg D., Fuchs D., Gunther R , Hausen A., Werner E R. and Wachter H.: Urinary neopterin reflects clinical activity in patients with rheumatoid arthritis *Arthritis Rheum.* **29** (1986) 1063–1070
7. Krause A., Protz H and Goebel K M. Correlation between synovial neopterin and inflammatory activity in rheumatoid arthritis *Ann. Rheum. Dis.* **48** (1989) 636–640.
8. Niederwieser D , Huber C., Gratwhol A., Bannert P., Fuchs D., Hausen A., Reibnegger G , Speck B. and Wachter H Neopterin as a new biochemical marker in the clinical monitoring of bone marrow transplant recipients *Transplantation* **38** (1984) 497–500
9. Niederwiesser D., Herold M., Woloszczuk W., Aulitzky W , Meister B , Tilg H., Gastl G., Bowden R and Huber C. Endogenous IFN-gamma during human bone marrow transplantation *Transplantation* **50** (1990) 620–625
10. Troppmair J., Nachbaur K., Herold M., Aulitzky W., Tilg H., Gastl G., Bieling P , Kotlab B., Flener R., Mull B., Aulitzky W. O., Rokos H. and Huber C.: *In vitro* and *in vivo* studies on the induction of neopterin biosynthesis by cytokines, alloantigens and lipopolysaccharide *Clin Exp Immun* **74** (1988) 392–397.
11. Henderson D. C , Sheldon J., Riches P and Hobbs J. R Cytokine induction of neopterin production *Clin Exp Immun* **83** (1991) 479–482
12. Munker R., Norman A. and Koeffler H P Vitamin D compounds. Effect on clonal proliferation and differentiation of human myeloid cells. *J. Clin. Invest.* **78** (1966) 424–30
13. Cohen M. S , Mesler D. E., Snipes R G. and Gray T K 1,25-dihydroxyvitamin D3 activates secretion of hydrogen peroxide by human monocytes. *J Immun.* **136** (1986) 1049–1053
14. Dodd R C , Cohen M S., Newman S L. and Gray T Vitamin D metabolites change the phenotype of monoblastic U937 cells *Proc Natn. Acad Sci U S A* **80** (1983) 7538–7541

15. McCarthy D., Hibbin J., San Miguel J. F., Freake H., Rodriques B., Andrews C., Pinching A., Catovsky D. and Goldman J. M.: The effect of vitamin D3 metabolites on normal and leukaemic bone marrow cells *in vitro*. *Int. J. Cell Clon* 2 (1984) 227–242.
16. Ossenkoppele G. J., Wijermans P. W., Nauta J. J. P., Hijgens P. C. and Langenhuijsen M. M. A. C.: Maturation induction in freshly isolated human myeloid leukaemic cells, $1,25(OH)_2D3$ vitamin D3 being the most potent inducer. *Leuk. Res.* 13 (1989) 609–614.
17. Kelsey S. M., Makin H. L. J., Macey M G. and Newland A. C.: Gamma interferon augments functional and phenotypic characteristics of vitamin D3-induced monocytoid differentiation in the U937 human leukaemic cell line *Leuk Res.* 14 (1990) 1027–1033.
18. Kelsey S. M., Makin H. L. J. and Newland A. C. : Functional significance of differentiation in human myeloid leukaemic blasts induced by 1,25-dihydroxyvitamin D3 and GM-CSF. *Leuk. Res.* (1992).
19. Goyert S. M., Ferrero E , Rettig W. J., Yenamandra A. K., Obata F. and LeBeau M. M.: The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. *Science* 239 (1988) 497–499.
20. Werner-Felmayer G., Werner E. R., Fuchs D., Hasen A., Reibnegger G. and Wachter H.: Neopterin formation and tryptophan degradation by a human myelomonocytic cell line (THP-1) upon cytokine treatment *Cancer Res.* 50 (1990) 2863–2867.
21. Ziegler I., Schott K., Lubbert M., Herrmann F , Schwulera U. and Bacher A : Control of tetrahydrobiopterin synthesis in T lymphocytes by synergistic action of interferon gamma and interleukin 2. *J. Biol. Chem.* 265 (1990) 17026–17030.
22. Kelsey S. M., Allen P. D., Razak K., Macey M. G. and Newland A. C.: Induction of surface tumor necrosis factor (TNF) expression and possible facilitation of surface TNF release from human monocytic cells by granulocyte-macrophage colony-stimulating factor or gamma interferon in combination with 1,25-dihydroxyvitamin D_3. *Exptl Hemat.* 21 (1993) 864–869