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Effects of hexadecylphosphocholine on thrombocytopoiesis

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Abstract

Hexadecylphosphocholine (HePC) is the first representative of the alkylphosphocholines, a novel group derived from the cytotoxic etherlysophospholipids. HePC shows a broad spectrum of antiproliferative effects in neoplastic cells *in vitro* and *in vivo*. HePC has been tested successfully in several clinical studies. One of the remarkable features of this compound has been the induction of a leucocytosis and a thrombocytosis in most of the patients receiving HePC systemically. In this paper, we have investigated the biological and molecular mechanisms by which HePC exerts this interesting effect. We found that HePC acts as an unspecific costimulator on human megakaryocytic proliferation in a soft agar assay system predominately together with thrombopoietin (TPO). Furthermore, HePC leads to the synthesis and secretion of several haematopoietic growth factors in monocytes and bone marrow fibroblasts, determined by the direct measurement of growth factors in cellular supernatants and by the measurement of growth factor mRNA in cell extracts. Thus, HePC seems to produce the increase of blood platelets in tumour patients by two different mechanisms.

Keywords: Hexadecylphosphocholine; Alkylphosphocholines; Ether lipids; Thrombocytopoiesis

1. Introduction

Hexadecylphosphocholine (HePC) is the first compound of a new group of cytotoxic ether lipid-derived substances, the alkylphosphocholines (ALP). HePC has been investigated in a variety of human tumour cell lines *in vitro* and *in vivo* and in some chemically-induced rat tumour models [1–5] where it showed excellent antiproliferative activities. In several phase II/III investigations, HePC was tested in patients with mammary carcinoma [6,7], colorectal carcinoma [8,9], non-small cell lung carcinoma [10] and cutaneous lymphoma [11]. In 1992, HePC was approved as a drug in Germany for the topical treatment of metastasised mammary carcinoma.

One of the remarkable features of this compound is the fact that it shows a high degree of selectivity towards neoplastic cells, while hardly affecting normal tissues. Besides its antiproliferative effects, HePC can induce differentiation and apoptosis in malignant cell lines [12–14] and it inhibits the invasive growth of neoplastic cells into normal tissue [15]. In peripheral lymphocytes, HePC acts as a costimulant with interleukin-2 (IL-2) to induce a high expression of the IL-2 receptor and human leucocyte antigen-DR (HLA-DR) antigens on the cell surface [16]. In mononuclear blood cells, HePC enhances the synthesis and secretion of Interferon (IFN)-gamma and granulocyte macrophage-colony stimulating factor (GM-CSF) triggered by IL-2 [17].

The molecular mechanisms by which HePC induces its biological effects are still unclear. In recent years, several potential modes of action have been suggested [18–20]. Probably the most likely mechanism may be an interference with cellular signalling via phospholipases, mitogen-activated protein (MAP) kinase or the stress-activated protein (SAP) kinase/cJun kinase pathways [21–24].

A consistent observation in clinical studies with systemic oral application of HePC was a substantial increase of peripheral blood neutrophil granulocytes and platelets in most of the patients [25]. This effect, in particular, contrasts with the usual side-effects of a conventional cytotoxic therapy, where a depression of bone marrow is one of the main problems of the treat-

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ment procedure. In this paper, we therefore investigated the biological and molecular mechanisms underlying the increase of blood platelets following HePC therapy. We used primary *in vitro* cultures of human megakaryocytic precursor cells, bone marrow fibroblasts and peripheral blood monocytes to test the effect of HePC on cell proliferation and the synthesis of haematopoietic growth factors.

2. Materials and methods

2.1. Reagents

HePC was obtained from Asta Medica, Frankfurt, Germany. Cell culture reagents were from Gibco, Paisley, Scotland, UK. Erythropoietin (EPO) and GM-CSF were from Dako, Glostrup, Denmark. IL-3, IL-6 and stem cell factor (SCF) were from Sigma, Munich, Germany. Thrombopoietin (TPO) was from R&D Systems, Wiesbaden, Germany.

2.2. Cell isolation and culture

Megakaryocytic precursor cells were isolated from bone marrow specimens obtained from voluntary donors undergoing sternotomy for an arterio-venous bypass operation. Bone marrow was first passed through a sterile metal filter in phosphate-buffered solution (PBS). The resulting cell suspension was washed twice in PBS and passed over a Nyco Prep gradient (Nycomed Pharma, Norway) with a specific density of 1.068 and centrifuged for 40 min at 600*g*. The cell sediment was incubated with a monoclonal mouse antibody directed against human cluster designation (CD)61 antigen (Gp IIIa, Dako, Denmark). The cells were washed again and incubated with 10 μg/ml of an iron-particle coupled goat-antimouse antibody. The cell suspension was then passed over a magnetic separation column (MAC Magnetic Microbeads, Miltenyi Biotec, Germany). CD61 positive cells attached to the column were rinsed off after removing the magnet. The purity of this preparation reached 95% for the megakaryocytic precursors as determined by fluorescence-activated cell sorting (FACS) analysis with an anti-CD41a antibody (Dako, Denmark). The cells were counted and then seeded into six well plates in 3 ml of methylcellulose/Iscove's modified Dulbecco's medium (IMDM) medium enriched with fetal calf serum (FCS) (9/1 (v/v)). If not otherwise stated, haematopoietic growth factors were added at the following concentrations: IL-3 1 ng/ml, IL-6 30 ng/ml, GM-CSF 100 ng/ml, SCF 20 ng/ml and TPO 50 ng/ml. HePC was added at a concentration of 0.5 μg/ml. Cells were cultured at 37°C and 5% CO_2 for 10 days and the cell number was determined thereafter by cell counting in a Neubauer chamber.

Bone marrow fibroblasts were isolated by passing a part of the marrow over a Ficoll gradient (Seromed, Germany, 1.077) and cultivating the cell suspension in 10 ml IMDM medium with horse serum (9/1 (v/v)) at 37°C in plastic culture flasks. The medium was changed daily until all non-adherent cells were removed and the bottom of the flask was covered by fibroblasts. Monocytes from peripheral blood were isolated over a Nycomed gradient (1.068). The purity was checked by FACS-analysis using an anti-CD14 monoclonal antibody at a concentration of 5 μg/ml (Dako, Denmark) showing on average 86–90% CD14-positive cells.

2.3. Determination of haematopoietic growth factors

Growth factors released into the medium of HePC stimulated or unstimulated monocytes and bone marrow fibroblasts were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Germany) according to the recommendations of the manufacturer.

2.4. Determination of growth factor RNA

1×10^6 fibroblasts or monocytes were lysed and RNA was isolated by the Purescript RNA isolation kit (Bioenzyme Diagnostik, Germany). 5 μg of RNA were incubated with the corresponding commercially available hexamer random primer for *IL-6*, *IL-3* (Clontech Laboratories, USA), GM-CSF, tumour necrosis factor (TNF)-α (R&D Systems, Germany) and TPO (Delimel, Germany) for 10 min at 70°C and then put on ice. First-strand buffer, dithiothreitol (DTT), deoxynucleotide triphosphate (dNTP) (final concentration of 220 nmol/ml) and Monkey-Maloney leukaemia virus (M-MLV) reverse transcriptase (all Gibco BRL, Germany) were added followed by an 50 min incubation at 42°C and 10 min at 70°C. The resulting cDNA was then amplified by a polymerase chain reaction (PCR) in a thermocycler by addition of 5′ and 3′ primers and 34 U/ml Taq Polymerase (Gibco BRL, Germany).

The corresponding thermocycler conditions were for the *IL-3* and *IL-6* primers 5 min at 94°C for one cycle, denaturation for 30 s at 94°C, hybridisation 30 s at 64°C and elongation for 1 min at 72°C for a total of 35 cycles. Conditions for the *GM-CSF* primers were 5 min at 95°C for one cycle, denaturation for 45 s at 95°C, hybridisation for 30 s at 58°C and elongation for 30 s at 72°C for 30 cycles. For the *TNF-α* primers, conditions were 5 min at 95°C for one cycle, denaturation for 45 s at 95°C, hybridisation for 30 s at 60°C and elongation for 30 s at 72°C for 30 cycles. Conditions for the *TPO* primers were 5 min at 94°C for one cycle, denaturation for 30 s at 94°C, hybridisation for 30 s at 64°C, elongation for 2 min at 72°C for 36 cycles.

The amplified cDNA was separated electrophoretically in a 2% agarose-gel in presence of 1 μg/ml ethidium bromide. Specific DNA bands and standards were visualised under ultraviolet (UV) light and photographed by a Polaroid camera.

3. Results

3.1. Effects of HePC on the proliferation of megakaryocytic precursor cells

To test the effects of HePC on the *in vitro* proliferation of megakaryocytic precursors, these cells were isolated from bone marrow and maintained for 10 days in a methylcellulose-containing culture medium. The purity of the primary cell isolates and of the cells after cultivation was checked by FACS analysis or by alkaline phosphatase anti-alkaline phosphatase (APAAP) staining and showed an almost completely pure CD41a-positive population. Various mixtures of haematopoietic growth factors were added to the cultures always omitting one factor. Cell proliferation was measured by counting the cells after the incubation period of 10 days, comparing it with a culture containing only FCS. As positive controls, a culture with all the growth factors and a culture with a serum of a patient with aplastic anaemia was used. Fig. 1a–g illustrates the results of these experiments. A consistent observation was the rather high interindividual differences of cell proliferation between the tested bone marrow donors of approximately 20%. In some of the cultures with FCS alone, a decrease of the original cell count was apparent. Following the addition of HePC this effect could be neutralised to a certain degree and in some cultures a slight increase occurred. A clear stimulatory effect of HePC on megakaryocytic precursor cell proliferation was not detectable. The addition of aplastic serum or a complete mixture of haematopoietic growth factors induced a pronounced increase of cell number in all donors. This was further enhanced, to a small degree, by the addition of HePC. By omitting single growth factors it was shown that TPO had the strongest proliferation-inducing effect. Independent of whether the growth factors were present or missing, HePC always increased the cell count by approximately 5–25%. Thus, HePC seems to induce a rather unspecific costimulatory effect with haematopoietic growth factors involved in the proliferation of megakaryocytic precursor cells.

3.2. Effects of HePC on cytokine release in monocytes and fibroblasts

To investigate the effects of HePC on cells which are potentially involved in haematopoiesis by providing growth factors for haematopoietic precursors, we exposed monocytes from peripheral blood and bone marrow fibroblasts to 0.5 μg/ml HePC over time periods of 48 h and 8 days, respectively. Cytokine release of these cells was quantified by ELISA in the supernatants of the cell culture. In the experiments with monocytes, we employed as a positive control lipopolysaccharide (LPS), which is a well known activator of monocytic cells.

Figs. 2a–d show the results of these experiments. Already after a short period of time of 3–6 h HePC induced a substantial secretion of IL-6, TPO and GM-CSF. IL-3 and SCF could not be detected in any of the cultures (data not shown). The amounts of cytokines found in the medium differed between individuals to a high degree. For TPO between 70.7 and 160.0 pg/ml, for IL-6 24.1 and 102.4 pg/ml and for GM-CSF 8.9 and 23.9 pg/ml. Interestingly, HePC led to a similar intensity and kinetics of cytokine release as LPS as shown in Fig. 2d for IL-6. However, in contrast to LPS, there were test persons whose monocytes temporarily could not be stimulated with HePC (see Fig. 2c for GM-CSF). After some time, these donors became HePC susceptible again (data not shown).

The same experimental setting with bone marrow fibroblasts (Fig. 3a–d) revealed high concentrations of TPO, SCF, GM-CSF and IL-6 in the supernatant induced by HePC exposure. HePC-stimulated cells showed on average a 3–4-fold increase of cytokine amounts on day 8 compared with $t=0$. Maximal concentrations varied depending on the donor and were 51.1–125.4 pg/ml for TPO, 2.4–50.2 pg/ml for SCF, 2.5–35.3 pg/ml for GM-CSF and 74.5–895.1 pg/ml for IL-6. As before, in some donors distinct growth factors could not be induced by HePC.

3.3. Effects of HePC on cytokine mRNA synthesis

To finally test whether the secretion of haematopoietic cytokines in monocytes and bone marrow fibroblasts was only the result of cytokine release from intracellular stores triggered by HePC, we analysed the mRNA induction for the corresponding cytokines in both cell types under HePC treatment. Messenger-RNA from monocytes and bone marrow fibroblasts from the cultures treated with HePC for 3–24 h was extracted and transcribed into cDNA. This was amplified by PCR, separated electrophoretically and ethidium bromide stained bands were visualised under UV light. Fig. 4a–d shows the results in monocytes exposed to HePC. LPS-stimulated monocytes and *TNF-α* mRNA were used as controls. In all donors tested, mRNA for *IL-3* was not detectable, reflecting the lack of IL-3 secretion from the previous experiments. After 3 h of HePC or LPS exposure, the monocytes showed an induction of mRNA synthesis for *IL-6*, *GM-CSF*, *TPO* and *TNF-α*. The signal remained stable over 24 h. In the fibroblasts

(Fig. 5a–d), HePC also induced mRNA for *IL-6*, *GM-CSF* and *TPO* after 3 h of exposure. IL-3 was again negative. Thus, HePC stimulated the *de novo* synthesis and the secretion of haematopoietic growth factors involved in megakaryocytopoiesis in monocytes, as well as in bone marrow fibroblasts. Especially in fibroblasts, as shown in Fig. 3 the amount of growth factor compared with control was substantial (approximately 3–4-fold).

4. Discussion

HePC has been tested in several phase II clinical studies. The largest study with colorectal carcinoma showed only a minor response of the tumours which may have been due to the limited dose of HePC given to the patients because of gastrointestinal side-effects. Therefore, of particular interest was the observation

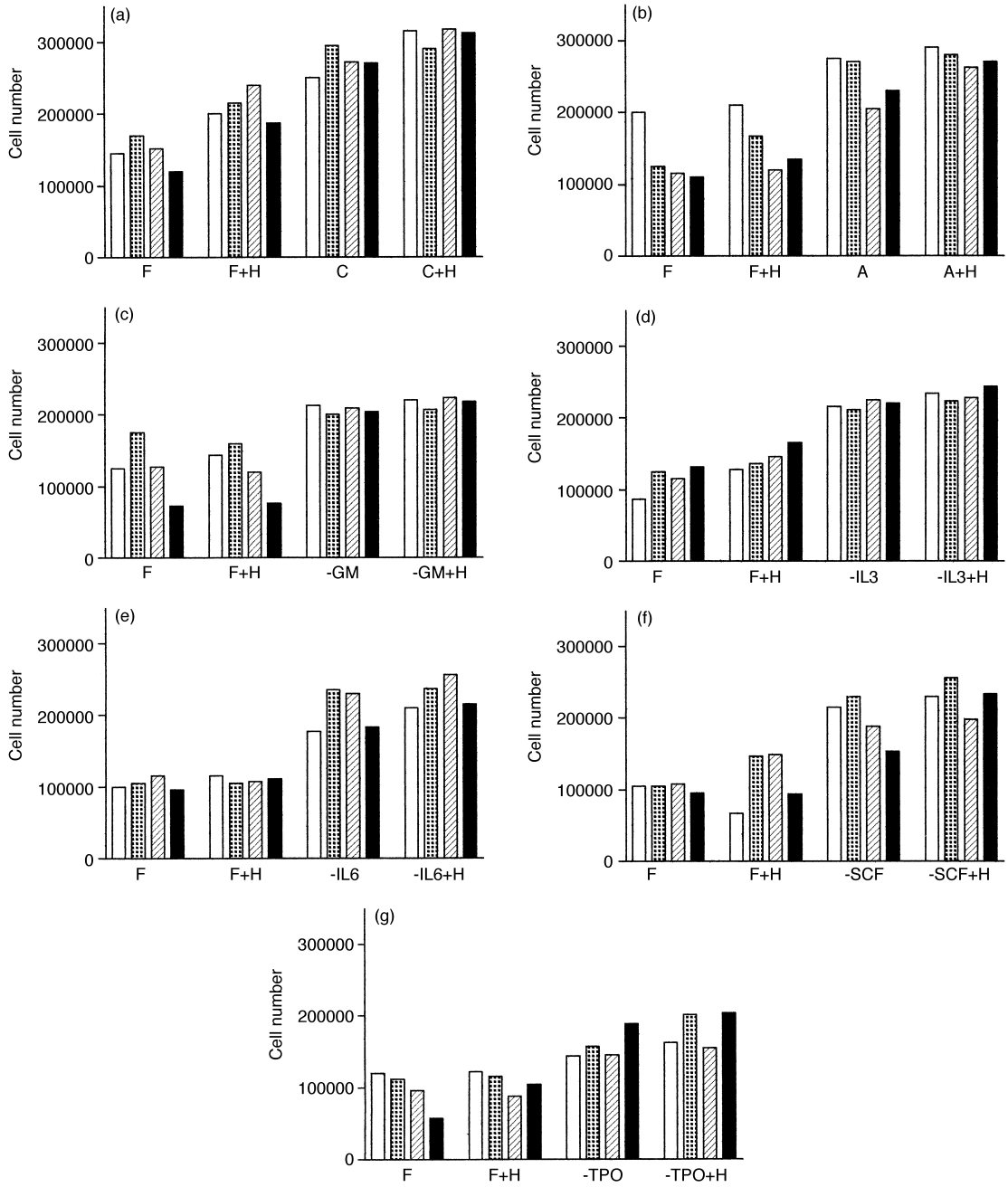

Fig. 1. Proliferation of megakaryocytic precursor cells. 10^5 megakaryocytic precursor cells from 4 different patients were incubated in methylcellulose-containing medium for 10 days. Cultures were maintained in presence of hexadecylphosphocholine (HePC) 0.5 μg/ml (H) and fetal calf serum (FCS) (F). Different mixtures of haematopoietic growth factors were added as described in the Methods: (a) mixture of stem cell factor (SCF), thrombopoietin (TPO), interleukin (IL)-6, IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) (C); (b) serum of patients with aplastic anaemia (A); (c) without GM-CSF (−GM): (d) without IL-3 (−IL3); (e) without IL-6 (−IL6); (f) without SCF (−SCF); (g) without TPO (−TPO).

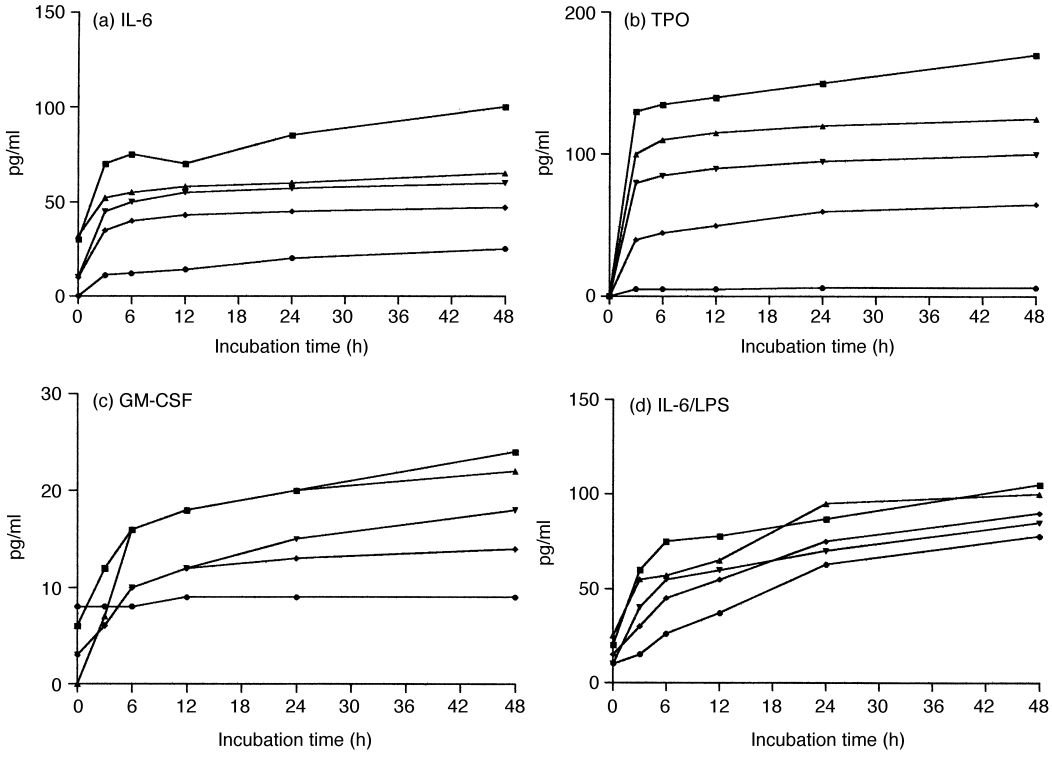

Fig. 2. Cytokine release in monocytes. 10^6 monocytes from peripheral blood specimens of five different donors were incubated for 48 h at 37°C in presence of 0.5 μg/ml of hexadecylphosphocholine (HePC). The concentration of growth factors in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) at the indicated time intervals: (a) interleukin (IL)-6; (b) thrombopoietin (TPO); (c) granulocyte macrophage-colony stimulating factor (GM-CSF); (d) the determination of IL-6 after stimulation of cells by lipopolysaccharide (LPS) alone in the absence of HePC.

that even in these low doses HePC produced no toxic effects on haematopoiesis. On the contrary, in 70% of the patients a rise of peripheral blood granulocytes and thrombocytes was noticed [9,25]. The maximal effect was observed during continuous therapy with HePC after 4 weeks. These authors speculated that HePC may induce an increase in peripheral cells by releasing cytokines. However, they could not detect any significant increase of G-CSF, GM-CSF, IL-1, IL-3 and IFN-γ levels in the serum of the patients.

In 1991, Vehmeyer and colleagues showed that HePC could stimulate the *in vitro* growth of haematopoietic precursor cells in a soft agar assay system. HePC had no direct proliferation-inducing effect, but costimulated the colony formation of myeloid precursors, together with granulocyte-colony stimulating factor (G-CSF), and to a lesser degree with GM-CSF [26]. The elimination of accessory cells had no effect on the effects of HePC in this system. These results provide a reasonable explanation for the increase of neutrophil granulocytes as G-CSF is the most important growth factor for these cells.

We investigated here whether similar mechanisms may be responsible for the increase of peripheral blood platelets. The treatment of megakaryocytic precursor cells with non-cytotoxic doses of HePC did not lead to any direct stimulatory effect on cellular proliferation. Together with growth factors, however, HePC showed significant costimulatory effects increasing the cell count for 5–25%. The dose range of HePC where these effects were observed was from 0.25 to 1 μg/ml. A further increase of the HePC concentrations led to inhibition of cell growth in this culture system (data not shown). Similar dual effects of stimulation at low and growth retardation at higher doses were also described for myeloid precursor cells [26]. In our assay system, TPO was the most potent stimulator of megakaryocytic proliferation, which is in accordance with previous observations. In contrast to the induction of myeloid cells, there was apparently no special growth factor with which HePC costimulated the precursor cells. It rather seems that HePC enhances the growth factor signalling in an unspecific and unselective manner.

Generally, we observed an increase of cell numbers of approximately 5–25%. This seemed not to be enough to explain the increase of platelets in peripheral blood. In contrast, Vehmeyer and colleagues [16] observed a 4- to 6-fold increase in colony formation in their investigations with myeloid precursors. A possible explanation for this discrepancy could be that HePC *in vivo* may activate other cells to produce more growth factors which in turn could then further increase the proliferation of megakaryocytes. Therefore, we tested the effects

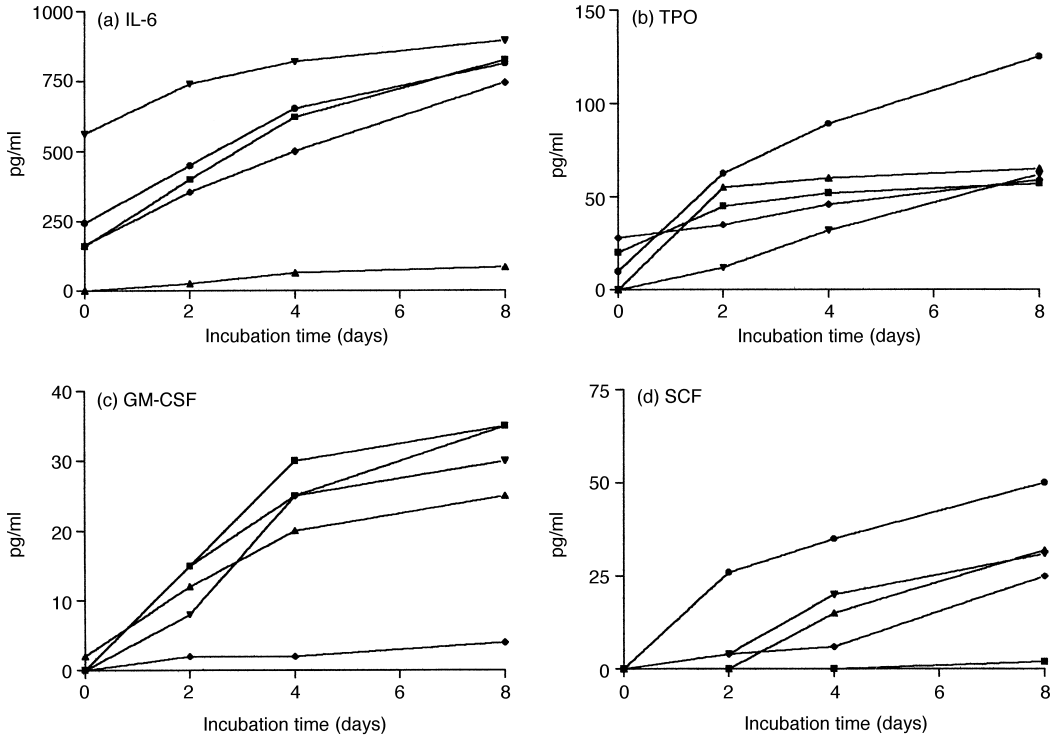

Fig. 3. Cytokine release in bone marrow fibroblasts. 10^6 bone marrow fibroblasts from five different donors were incubated for 8 days at 37°C in presence of 0.5 μg/ml hexadecylphosphocholine (HePC). The concentration of growth factors in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) at the indicated time intervals: (a) interleukin (IL)-6; (b) thrombopoietin (TPO); (c) granulocyte macrophage-colony stimulating factor (GM-CSF); (d) stem cell factor (SCF).

of HePC on monocytes and bone marrow fibroblasts. Interestingly, HePC was a potent activator of monocytes and bone marrow fibroblasts leading to a significant induction of synthesis and secretion of IL-6, TPO and GM-CSF. The potency of HePC-mediated cell activation was comparable to that of LPS, a classic activator of monocyte functions. Similar observations have already been made with other ether lipid derivatives. Platelet activating factor (PAF) and ET-18-OCH_3 can stimulate mouse peritoneal macrophages to produce TNF-α [27]. However, in these experiments cells had to be costimulated with LPS or thioglycolate. An activation of macrophages *in vivo* and *in vitro* by several different ether lipid analogues measured by a increased rate of phagocytosis was described for mouse peritoneal macrophages and human lung macrophages by Andreesen and coworkers [28]. To which extent these effects may play a role in the antitumoral activities of these substances still remains hypothetical, as adequate data are missing. The stimulating effect of HePC apparently is not restricted to one cell type as shown by the substantial stimulation of bone marrow fibroblasts.

A smaller degree of unspecific stimulation of precursor cell proliferation and a high degree of growth factor synthesis and release by fibroblasts and monocytes argues in favour of an indirect effect of HePC on megakaryocytopoiesis via accessory cells in the study patients. This finding is in contrast with the observations of Vehmeyer and colleagues [16] who ruled out an influence of accessory cells for the costimulatory effects of HePC on myeloid cell proliferation. There are two possible explanations for this discrepancy. First of all, the stimulation of megakaryocytic precursors by HePC may principally be different from that of other myeloid cells. Secondly, one has to keep in mind that the accessory cells in the experiments of this group have not been tested directly, but the lack of effects of these cells was assumed after they were eliminated from the cultures. The problem of this procedure is that a total elimination of accessory cells by the methods employed in these experiments probably cannot be achieved, even more so as the purity of the cell preparations has not been checked by appropriate methods. A small amount of accessory cells may be just sufficient to produce enough growth hormone to stimulate colony formation.

The fact that there seem to be individuals whose cells were temporarily not susceptible to stimulation by HePC perhaps explains the observation that only approximately 70% of patients reacted with an increase of peripheral platelets. It is still unclear what factors determine the state of unresponsiveness, especially as some donors in our experiments were only temporarily unresponsive, but became positive at later testing. Similar results were also obtained by Vehmeyer and colleagues in their investigations on the effects of HePC on granulocytopoiesis [16,26].

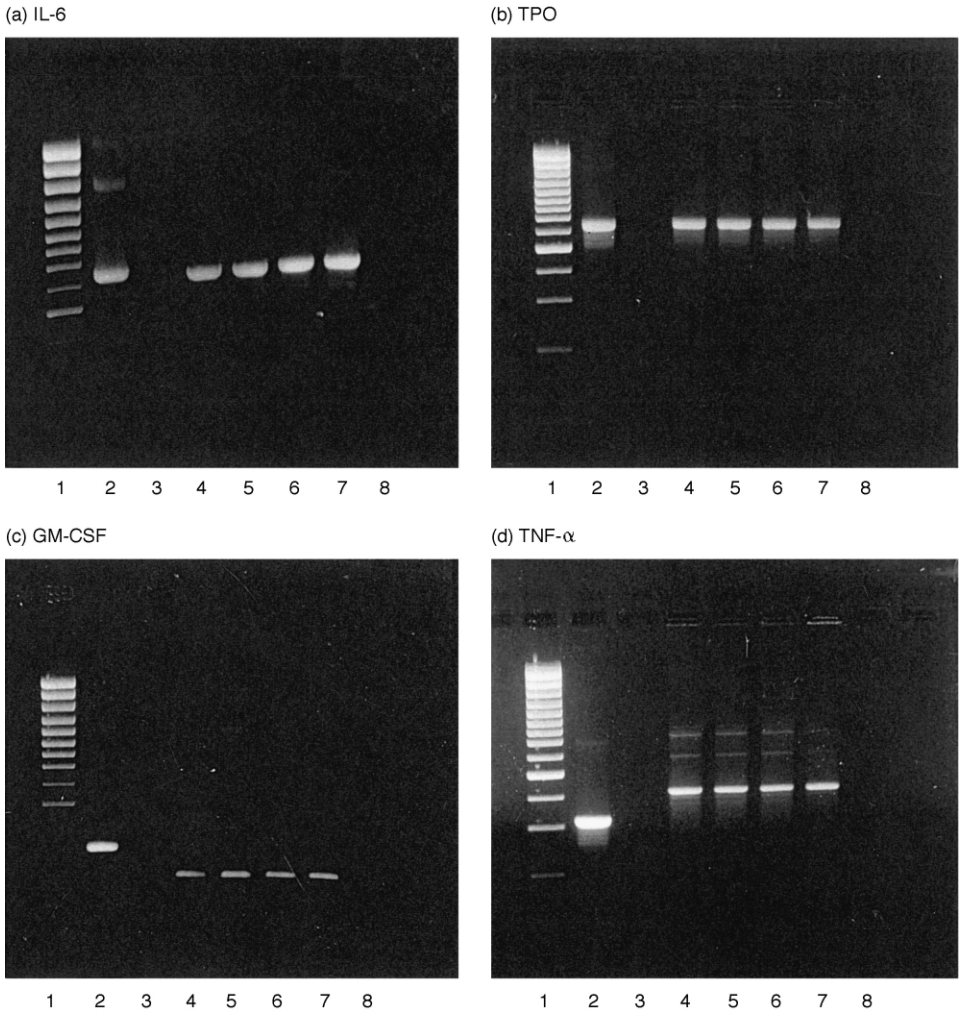

Fig. 4. Induction of haematopoietic growth factor mRNA in monocytes. 10^6 monocytes from peripheral blood were incubated in presence or absence of 0.5 μg/ml hexadecylphosphocholine (HePC) for 24 h at 37°C. At the indicated time intervals, RNA was extracted, transcribed into cDNA and amplified by PCR. The cDNA was separated by electrophoresis and visualised by ethidium bromide staining. Lane 1 shows the molecular markers, lane 2 is the positive control, lane 3 is the negative control, lanes 4–7 are specimens after 3, 6, 12 and 24 h of incubation with HePC and lane 8 is a specimen after 24 h of incubation without HePC.

Furthermore, it remains unclear by which mechanism HePC exerts its stimulatory and costimulatory effects on normal haematopoietic cells and fibroblasts. The rather unspecific stimulation of megakaryocytic precursors points at an equally unspecific molecular mechanism. Currently, it is considered that HePC and similar substances act inside the cell and influence membrane-dependent biological reactions. It is conceivable that HePC may change the presentation of growth factor receptors on the target cells. However, most of the membrane receptors investigated so far show consistently a downregulation of receptor molecules on the cell surface by HePC and ET-18-OCH_3 [23,29,30]. HePC and other ether lipids led to unspecific changes of membrane phospholipid composition, which may induce a proliferation stimulus in some cells. There are two other noteworthy characteristics of cell stimulation by HePC. First of all, the stimulation occurs only at relatively small doses of the compound. At higher concentrations, antiproliferative effects prevail. Secondly, a stimulation of neoplastic cell growth has so far not been described.

The fast and pronounced activation of monocytes and bone marrow fibroblasts indicates, in contrast to the effects on megakaryocytic precursors, a quite specific mode of action. This activation signal cannot be delivered by cell receptors as the activation through LPS via CD14, because there exist no specific binding sites for HePC and other ether lipid analogues except PAF [31,32]. HePC may, however, induce cell activation by modulating intracellular signal transduction. An interference with phospholipase A_2, C and protein kinase C, MAP kinase and SAP kinase/cJun NH_2-terminal kinase has been described [18,21–24].

Recently, it was shown that HePC is also a potent antiprotozoal agent against visceral leishmaniasis

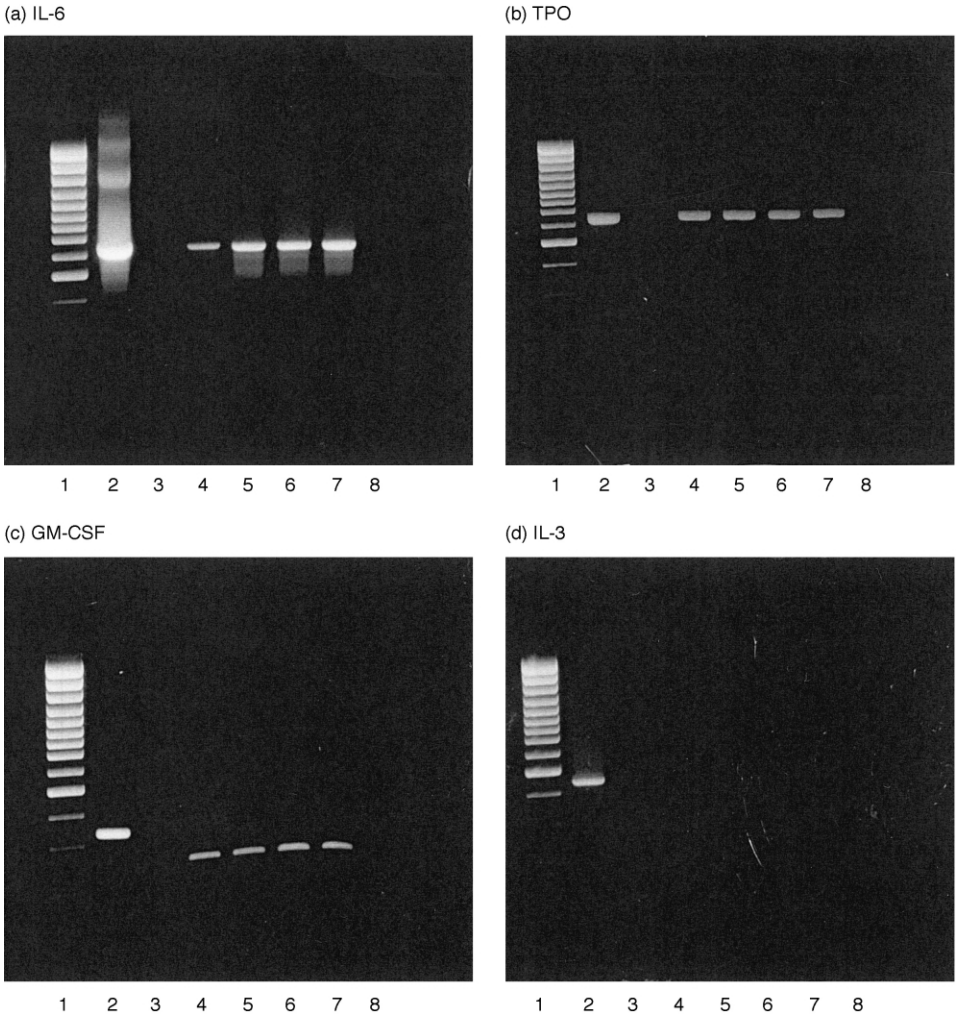

Fig. 5. Induction of mRNA for haematopoietic growth factors in bone marrow fibroblasts. 10^6 bone marrow fibroblasts were incubated in presence or absence of 0.5 μg/ml hexadecylphosphocholine (HePC) for 24 h at 37°C. At indicated time intervals, RNA was extracted, transcribed into cDNA and amplified by PCR. The cDNA was separated by electrophoresis and visualised by ethidium bromide staining. Lane 1 shows the molecular markers, lane 2 is the positive control, lane 3 is the negative control, lanes 4–7 are specimens after 3, 6, 12 and 24 h of incubation with HePC and lane 8 is a specimen after 24 h of incubation without HePC.

[33,34]. One could assume that this effect may be mediated by activation of T-cells and macrophages by HePC. However, Murray and colleagues showed that this antiprotozoal effect was also retained in genetically-defective mice, lacking T-cells, endogenous IFN-γ and macrophage-dependent leishmanicidal activity [35]. These findings indicate that most of the biological effects in different cell systems are probably resulting from the direct influence of HePC on some cellular functions, perturbation of membrane functions or cellular signalling.

This interesting biological effect of HePC on haematopoietic cells may be further developed and enhanced by structural changes of the original substance resulting in new compounds with predominately cell-stimulatory characteristics, fewer side-effects and more favourable pharmacological properties. Thus, it is conceivable that such substances could be used in future to alleviate perhaps the bone marrow toxicity of the classic antineoplastic drugs. Structure–effectiveness studies with these derivatives may also help us to learn more about the precise mechanisms by which HePC and similar compounds exert their pleiotropic effects.

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