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Cytokine-mediated Inhibition of Erythropoietin Synthesis by Dexamethasone

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Abstract

The effect of inflammatory cytokines on the in-vitro synthesis of erythropoietin by HepG2 cells was evaluated.

Monocyte-conditioned medium, and the cytokines interleukin-1β, interleukin-6 and tumour necrosis factor-α all reduced synthesis of erythropoietin. The steroidal anti-inflammatory drug, dexamethasone, did not affect cytokine-mediated erythropoietin inhibition. Dexamethasone did cause a reduction in the secretion of erythropoietin inhibitory cytokines from monocytes.

These results point to a possible therapeutic approach in the treatment of anaemia caused by suppression of erythropoietin synthesis by monocytic cytokines.

Anaemia commonly develops in patients with chronic inflammation caused by infectious diseases. It is now believed that anaemia develops in response to release of cytokines from monocytes in the inflammatory process. These cytokines either inhibit the biosynthesis of erythropoietin or antagonize the stimulatory action of erythropoietin on erythroid precursor cells in the bone marrow.

Erythropoietin is a glycoprotein hormone essential for regulation of erythropoiesis. It is produced in adults mainly by the kidney and during foetal life by the liver. It acts by binding to specific receptors on erythroid progenitor cells, inducing their proliferation and differentiation into mature red blood cells.

The cytokines interleukin-1 and tumour necrosis factor-α (TNFα) are inflammatory mediators implicated in anaemia of chronic diseases.

The aim of this study was to determine the individual effects of monocyte conditioned medium (MCM), IL-1, IL-6, TNFα and neopterin on in-vitro production of erythropoietin.

Materials and Methods

Reagents

Human recombinant IL-1β was provided by the Biological Resources Branch of the Biological Response Modifiers Program, Division of Cancer Treatment/United States National Cancer Institute (Frederick, MD, USA). Recombinant human IL-6 and TNFα were purchased from Boehringer Mannheim (South Africa Pty. Ltd). Foetal calf serum was obtained from Highveld Biological Pty. Ltd (Kelvin, SA), and trypsin, thioglycollate broth, phosphate-buffered saline (PBS), HEPES buffer and minimal essential medium (MEM) from Gibco (Grand Island, New York, USA). Dexamethasone and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sac-cel (donkey anti-rabbit antibody-coated cellulose suspension) was obtained from Immunodiagnostic Systems (UK). ^{125}Iodinated erythropoietin (370 kBq, 10 μg 0·5 mL^{-1}) was purchased from Amersham International Plc, (Amersham, UK). All chemicals were of analytical grade.

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Cells and cell culture

HepG2 cells were a gift from Dr Barbara B. Knowles (Wistar Institute, PA, USA). The cells were cultured in MEM, supplemented with 10% foetal calf serum (not heat-inactivated), in humidified air with 5% CO_2 at 37°C. Near-confluent cultures were passaged once weekly by treatment with 0·25% trypsin and 0·02% EDTA, in calcium- and magnesium-free PBS.

Preparation of conditioned medium

Inflammatory monocytes were induced in five male Balb/c mice (20 g) by intraperitoneal injection of 2 mL 3% thioglycollate broth. Seventy-two hours later the monocytes were collected by peritoneal lavage using PBS containing 2% heat-inactivated foetal calf serum. The cells were pooled and centrifuged at 200 *g* for 10 min at 4°C, and washed once with MEM containing 5% foetal calf serum and gentamicin (20 μg mL^{-1}).

Adherent monocytes were obtained by plating the cells in Falcon plastic 75-cm^2 flasks (Becton Dickinson, Oxford, UK) for 2 h at 37°C in 5% $C0_2$/95% air, followed by removal of nonadherent cells by suction. One half of the monocyte cultures were incubated with MEM, supplemented with 10% foetal calf serum and 10 μg mL^{-1} LPS, and the other half with the same medium containing in addition 1 μM dexamethasone. The cells were cultured for 18 h under standard conditions at a density of 10^6 cells mL^{-1}.

Dose-response relationships

HepG2 cells were plated 5 × 10^5 cells/well in 24-well Falcon culture plates (Becton Dickinson). Following adherence, after overnight incubation, medium was replaced by 1 mL standard culture medium containing a specific dose of test substance. Cells were cultured in this medium for a further 24 h, after which the medium was transferred to Eppendorf tubes (Lasec,

Cape Town, SA), and stored at −40°C for erythropoietin analysis. Cell monolayers were washed with PBS and the cells lysed with 0·5 mL NP 40 lysis buffer (0·5% NP 40, 90 mM KCl, 1 mM magnesium acetate, 10 mM HEPES, and 2 mM mercaptoethanol). Protein analysis was carried out on a 1 in 10 dilution (with PBS) of the cell lysates using the BioRad micro assay kit (BioRad Laboratories, UK). Each dose of test substance was assayed in quadruplicate, and each experiment was repeated three times.

Cytokine combination assays

Synergism between the test agents on erythropoietin synthesis was determined by incubating HepG2 cells with doses of 100 units mL^{-1} of each in the following combinations: IL-1β and IL-6, IL-1β and TNFα, IL-6 and TNFα, and IL-1β, IL-6, and TNFα. In addition, the effects of each of the cytokines on HepG2 erythropoietin secretion were tested in the presence of dexamethasone (1 μM) and 10% MCM.

Erythropoietin assay

Erythropoietin concentrations in cell culture were measured by radioimmunoassay (RIA), using a modified method of Randall et al (1993). To 100 μL standard or sample in 5-mL plastic tubes, 200 μL rabbit anti-human erythropoietin antiserum was added and maintained for 72 h at 4°C. Two hundred microlitres of ^{125}I-erythropoietin(10 000 counts min^{-1}) was then added and incubated for a further 24 h at 4°C. One hundred microlitres of Sac-cel was added to all tubes, except the total activity tubes; the mixture was allowed to stand at room temperature for 30 min, after which 1 mL distilled water was added. The tubes containing Sac-cel were centrifuged for 10 min at 1500 *g* at 4°C. Supernatant fluid was decanted, and the radioactivity in the pellets and total activity tubes was counted in duplicate in a Crystal II multidetector RIA system (Packard Instruments, USA). A standard curve was drawn using the logistic fit of the instrument, from which the results were calculated.

Statistical analysis

One-way analysis of variance was used to analyse the dose-response curves. Differences between doses were compared using multiple-range analysis.

Comparisons of the effects of single doses of test substances alone and in combination were carried out using Dunnet's *t*-test. Data are expressed as means ± s.e.m.

Results

Cytokine-mediated effects

Concentration-effect curves for IL-1β, IL-6, TNFα and neopterin on erythropoietin secretion are presented in Fig. 1. Interleukin-1β was the most potent, having a half maximum inhibitory concentration (IC50) of 5 units mL^{-1}. The IC50 for TNFα, by comparison, was 70 units mL^{-1}. Interleukin-6 was the least potent inhibitor. An IC50 value for IL-6 could not be obtained since the highest dose (1000 units mL^{-1}) produced only a 44% reduction in erythropoietin secretion. Neopterin had no inhibitory effect. Potentiation was found between IL-1β and TNFα (Table 1), but not between the other combinations. The inhibition of erythropoietin secretion obtained with IL-1β and TNFα combined was more than with each cytokine alone ($P < 0·05$). All three recombinant cytokines that were tested augmented the inhibitory effect of MCM on erythropoietin production (Table 2).

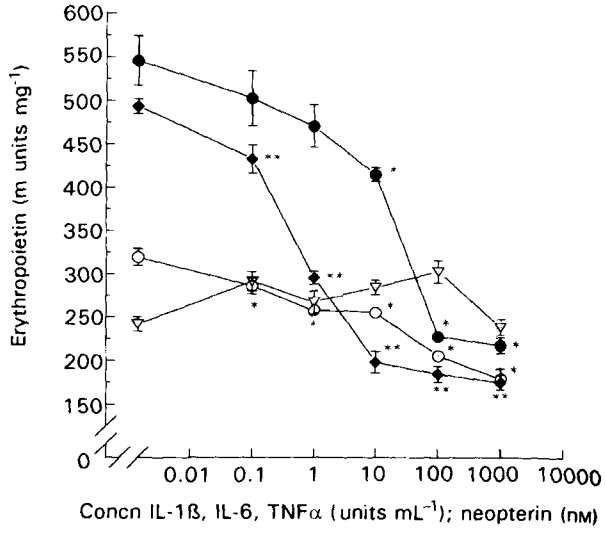

FIG. 1. Dose response curves of IL-1β(◆), IL-6 (○), TNFα(●) and neopterin (▽). Each point is the mean ± s.e.m. of four replicates. Each result is representative of three independent experiments. *$P < 0·05$, **$P < 0·01$ compared with control (first dose).

Table 1. Additive effects of IL-1β, IL-6 and TNFα on erythropoietin production by HepG2 cells.

	Erythropoietin (m units mg^{-1})
Control	495·4 ±19·04
IL-1β	144·4 ±7·54
IL-6	375·53 ± 17·92
TNFα	174·61 ± 13·06
IL-1β + IL-6	151·6 ±7·58
IL-1β + TNFα	98·15 ± 5·58
IL-6 + TNFα	174·73 ± 13·06
IL-1β + IL-6 + TNFα	129·07 ± 4·18

Table 2. The effects of IL-1β, IL-6 and TNFα on MCM-mediated erythropoietin suppression.

	Erythropoietin (m units mg^{-1})
Control	478.82 ± 9.96
MCM	265.74 ± 4.05
MCM + IL-1β	171.32 ± 10.02
MCM + IL-6	161.49 ± 3.5
MCM + TNFα	152.92 ± 2.04
MCM + IL-1β + IL-6 + TNFα	85.16 ± 1.5

Dexamethasone effects on cytokine-induced inhibition

Dexamethasone had no direct effect on cytokine-mediated inhibition of erythropoietin synthesis by HepG2 cells (Table 3). However, MCM obtained from dexamethasone-treated monocytes caused significantly less inhibition in HepG2 erythropoietin secretion than MCM from untreated monocytes ($P < 0·05$) (Fig. 2). It appears that dexamethasone reduces the secretion of erythropoietin-inhibitory cytokines from LPS-stimulated monocytes in culture.

Table 3. The effect of dexamethasone (1 μM) on the inhibition in erythropoietin synthesis caused by IL-1β(100 units mL^{-1}), IL-6 (100 units mL^{-1}) and TNFα (100 Units mL^{-1}).

Erythropoietin modulators	Erythropoietin secretion (m units mg^{-1})
	144.40 ± 7.54
IL-1β	132.43 ± 3.04
IL-1β + dexamethasone	265.80 ± 8.78
IL-6	256.95 ± 19.02
IL-6 + dexamethasone	174.61 ± 13.06
TNFα	183.55 ± 4.12
TNFα + dexamethasone	

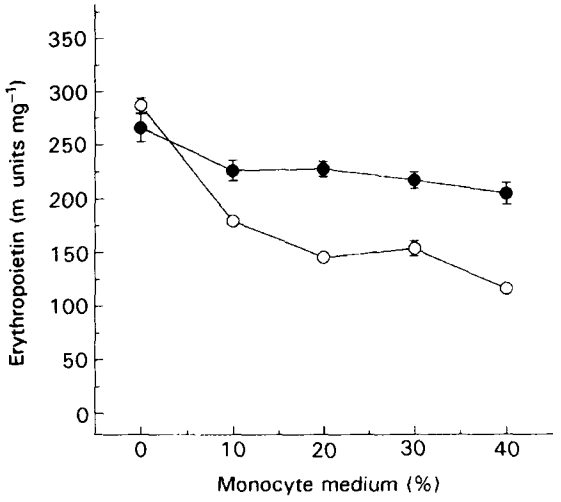

FIG. 2. Dose-response effects of MCM from dexamethasone-treated (●) and untreated (○) monocytes.

Discussion

The results show that cellular synthesis of erythropoietin is depressed by the inflammatory cytokines IL-1β, TNFα and, to a lesser extent, IL-6. This confirms in part the finding of Jelkmann et al (1990) that IL-1β and TNFα, but not IL-6, reduce erythropoietin output by HepG2 cells. The failure of Jelkmann et al (1990) to demonstrate inhibition in erythropoietin synthesis with IL-6 could be due to a lack of sensitivity of their assay, particularly since the decrease in erythropoietin synthesis we observed with IL-6 was not as pronounced as that with IL-1β and TNFα. Faquin et al (1992), using Hep3B cells grown under hypoxic conditions (1% O_2) to stimulate erythropoietin output, found that IL-6 reverses the inhibition in erythropoietin production by IL-1β and TNFα. In our study, which was carried out under normoxic conditions (21% O_2) and reflects constitutive erythropoietin production, IL-6 inhibited the synthesis of the hormone, but did not potentiate or reverse the inhibition caused by IL-1β and TNFα. These differences may, thus, be attributable to experimental conditions. Interleukin-1β and TNFα inhibited erythropoietin synthesis additively.

All three cytokines tested potentiated the inhibitory action of MCM obtained from LPS-stimulated monocytes. The magnitude of the potentiation was similar in each case, suggesting that monocytes secrete other factors, besides IL-1β and TNFα, which depress erythropoietin synthesis and which are enhanced by IL-6. Dexamethasone, a potent anti-inflammatory glucocorticoid, had no direct effect on the inhibition of erythropoietin synthesis induced by IL-1β, IL-6 or TNFα. Glucocorticoids are essential for optimum regulation of acute phase protein synthesis by these cytokines (Andus et al 1988). In HepG2 cells, dexamethasone was found to enhance the action of these cytokines on the expression of acute phase genes (Baumann et al 1987) which suggests that this cell line possesses glucocorticoid receptors. The lack of effect of dexamethasone on cytokine-mediated erythropoietin suppression suggests that the mechanism by which they modulate erythropoietin synthesis is different to that by which they control the production of acute phase proteins. This may also suggest that erythropoietin is not an acute phase reactant. Conditioned medium from dexamethasone-treated monocytes almost completely reversed the reduction in erythropoietin secretion caused by MCM from untreated monocytes. Monocytes are the main source of inflammatory cytokines such as IL-1β, IL-6, and TNFα (Arai et al 1990). This suggests that both the expression and secretion of erythropoietin-inhibitory cytokines by monocytes are downregulated by dexamethasone. Glucocorticoids have been shown to suppress the transcription of cytokine genes such as IL-1 (Lew et al 1988), IL-6 (Tobler et al 1992) and TNF (Beutler et al 1986).

The usefulness of recombinant human erythropoietin against anaemia in chronic disorders has been shown in clinical trials (Abels 1992; Henry 1993). The fact that erythropoietin synthesis is inhibited by inflammatory cytokines, and the demonstrated inhibition of bone marrow erythropoiesis by IL-1β (Schooley et al 1987) and TNFα (Means & Krantz 1993; Rusten & Jacobsen 1995) suggests that glucocorticoids such as dexamethasone may be effective in the treatment of anaemia of chronic disorders.

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