Erythropoietin and interleukin-1β modulate nitrite production in a Swiss 3T3 cell model of rheumatoid synovial fibroblasts

S. Baig, Y. Patel, P. Coussons1 and R. Grant1

Department of Sport, Exercise and Biomedical Science, University of Luton, Park Square, Luton, Bedfordshire LU1 3JU, U.K.

Abstract

Erythropoietin (EPO), a haemopoietic growth factor and a primary regulator of erythropoiesis, is widely used to treat anaemia in various chronic complications of rheumatoid arthritis (RA). Fibroblast-like cells, found in the pannus tissue of joints, are thought to contribute to the inflammatory pathology of RA. Thus for the current study we investigated the effects of recombinant human EPO (rHuEPO) on NO metabolism, using an interleukin-1β (IL-1β)-stimulated Swiss 3T3 fibroblast monolayer as a model for fibroblast activity in RA. The results show that, over 3 days, both alone and in combination with the pro-inflammatory cytokine IL-1β (10 ng/ml), rHuEPO (25 μ-units/ml) induced significant production of nitrite in cell culture supernatants. This is an indicator of NO production by nitric oxide synthase (NOS), which is a well-documented mediator of metalloproteinase-mediated tissue remodelling in RA. It therefore appears that, through modulation of NOS-dependent NO production, rHuEPO may influence remodelling of connective tissue in RA, independently of its established erythropoietic role.

Introduction

Rheumatoid arthritis (RA) is a persistent autoimmune disease, involving chronic inflammation of the diarthroidal joints, which causes cartilage degradation and bone destruction [1]. Synovial fibroblasts are a major component of rheumatoid pannus tissue, which on interaction with endothelial cells and macrophages produce chemical mediators, including NO, pro-inflammatory cytokines and matrix metalloproteinases [2]. These compounds function collectively to modulate inflammation and joint destruction in RA.

Key words: anaemia, nitric oxide, rheumatoid arthritis.

Abbreviations used: EPO, erythropoietin; rHuEPO, recombinant human EPO; RA, rheumatoid arthritis; IL-1β, interleukin-1β; NOS, nitric oxide synthase; ACD, anaemia of chronic disease; L-NAME, N-(G)-nitro-L-arginine methyl ester.

NO is produced in response to inflammatory cytokines, particularly interleukin-1β (IL-1β), tumour necrosis factor α and interleukin-6 [3], which are thought to contribute to the pathogenesis of anaemia of chronic disease (ACD), and is a common feature of RA [4]. Since NO is a free radical that is capable of oxidative pathology, the presence of nitrotyrosines in patients with RA is consistent with the formation of peroxynitrite in vitro [5].

Erythropoietin (EPO), a 30.4 kDa haemopoietic growth factor, functions as a regulator of erythropoiesis [6] and is administered as therapy for the treatment of ACD in RA. At present little is known regarding the effect of recombinant human EPO (rHuEPO) on the inflammatory response associated with RA.

Swiss 3T3 fibroblasts have been shown to produce NO when stimulated with the pro-inflammatory cytokine IL-1β [7]. Thus, this cell line was selected for the current investigation into the effect of rHuEPO on NO release in a model of rHuEPO therapy of RA.

Materials and methods

Chemicals and reagents

All chemicals, medium and sterile 24- and 96-well plates were purchased from Sigma-Aldrich, unless otherwise stated. IL-1β was from Peprotech EC.

Cell culture

Swiss 3T3 fibroblasts were grown to 80% confluence in a monolayer in complete supplemented medium prior to the addition of various single or combined treatments as follows: rHuEPO (0–100 μ-units/ml) and/or IL-1β (0–20 ng/ml). The nitric oxide synthase (NOS) inhibitor N-(G)-nitro-L-arginine methyl ester (L-NAME; 100 μM) was applied in parallel to treatment groups as a control for NO production. The media and treatments were replenished every 24 h for up to 3 days. Media supernatants were stored in labelled Eppendorf microcentrifuge tubes at −20 °C until required for nitrite analysis. Cell viability was assessed using the Trypan Blue exclusion assay [8] and was found to be > 90% in all groups.
Measurement of nitrite
Nitrite production was measured in cell culture media using the Griess reaction [9]. The absorbance was measured at 540 nm and the nitrite concentration was determined from a sodium nitrite standard curve. Nitrite was not detected either in media blanks incubated for 96 h or in media prior to cell culture.

Statistical analysis
Data were analysed and compared with controls using a two-tailed Student’s t test. Significance was denoted as **P < 0.01 or ***P < 0.001.

Results
Swiss 3T3 fibroblasts that had been exposed to 10 and 20 ng/ml IL-1β for 24 h produced between 1 and 2 μmol/ml nitrite, following 1, 2 and 3 days of culture. Peak nitrite production was measured on exposure to 10 ng/ml IL-1β after 1 day of culture (Figure 1). This treatment increased nitrite production by 300–400%, compared with untreated control cells (P < 0.001). This effect was reduced to below the basal level of untreated cells following treatment with L-NAME (100 μM).

Unexpectedly, exposure of cells to rHuEPO (25 μ-units/ml) also induced nitrite production (Figure 2, upper panel). Although rHuEPO-induced nitrite production was much less than IL-1β-induced production, the increase of 10–15% compared with controls was statistically significant (P < 0.001). Exposure of cells to higher concentrations of rHuEPO (50–100 μ-units/ml) also caused an increase in nitrite production following 2 and 3 days of culture (Figure 2, upper panel), although these increased values did not achieve statistical significance compared with controls.

Further evidence that rHuEPO induced NO production in fibroblasts is the observed increase in nitrite production by cells co-treated with IL-1β and rHuEPO compared with cells treated with IL-1β alone. This increase was not statistically significant, but followed the same general pattern of an increase in nitrite production of 0.1–0.2 μmol/ml after 1, 2 and 3 days of cell culture (Figure 2, lower panel). The magnitude of this response was comparable with that measured following treatment of cells with rHuEPO alone (Figure 2, upper panel).

Discussion
IL-1β induces nitrite production, enhances the synthesis of human synovial fibroblasts and activates synoviocytes to synthesize and release collagenase, all modulators of matrix remodelling in RA [1]. Our findings show that, in a cell model of rHuEPO therapy of RA, direct exposure of Swiss 3T3 fibroblasts to 25 μ-units/ml rHuEPO, either alone or in combination with IL-1β, induced a significant increase in nitrite production in the surrounding culture medium. Inhibition of nitrite production to below basal levels by L-NAME, a non-specific NOS inhibitor, showed that nitrite production was derived from NOS activation.

Several factors from this study support the hypothesis that the treatment of Swiss 3T3 fibro-
Connective Tissue

blasts with rHuEPO induced NO production by approx. 10–15%. First, treatment of cells with rHuEPO alone produced a statistically significant increase in nitrite production. Secondly, although not statistically significant, the co-treatment of cells with rHuEPO plus IL-1β resulted in a consistent increase in nitrite production over that seen following treatment with IL-1β alone. Thirdly, the magnitude of this increase (0.1–0.2 μmol/ml) was comparable with that seen in cells treated with rHuEPO alone. This effect was consistently observed following treatment of cells with rHuEPO plus IL-1β after 1, 2 and 3 days of culture (Figure 2, lower panel).

Finally, further evidence that rHuEPO can induce NO production comes from recent findings in this laboratory, which show a statistically significant increase in nitrite production following exposure of porcine nasal cartilage explants to 25 μ-units/ml rHuEPO (S. Baig, Y. Patel, P. Coussons and R. Grant, unpublished work).

To summarize, rHuEPO is used to treat anaemia in RA. However, the precise effects of this treatment in arthritic inflamed joints have hitherto not been investigated. The current study demonstrates that rHuEPO may influence connective tissue remodelling in RA through the production of NO. Furthermore, rHuEPO may have additive effects with cytokines such as IL-1β. Thus, paradoxically, while effectively treating the anaemic symptoms of RA, there is a possibility that rHuEPO may potentiate joint inflammation. These findings pose the question of whether the most effective anti-anaemic dose of rHuEPO in RA therapy is necessarily the most effective treatment from the inflammatory point of view.

**Figure 2**

**Effects of EPO on nitrite production**

Upper panel: time course of nitrite production by Swiss 3T3 fibroblasts (n = 6) in tissue culture supernatant was measured during a 3 day period at 24 h intervals following treatment with 25, 50 and 100 μ-units/ml EPO. Lower panel: nitrite production was measured (n = 8) following treatment with 25 μ-units/ml EPO, alone and in combination with 10 ng/ml IL-1β. For statistical details see the text.
Tumour necrosis factor α up-regulates protein kinase R (PKR)-activating protein (PACT) and increases phosphorylation of PKR and eukaryotic initiation factor 2-α in articular chondrocytes

S. J. Gilbert1, V. C. Duance and D. J. Mason
Connective Tissue Biology Laboratories, School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, Wales, U.K.

Abstract
Our previous analysis of the genes regulated in cartilage at the onset of spontaneous osteoarthritis in the guinea pig knee revealed up-regulation of the gene for protein kinase R (PKR)-activating protein (PACT), which encodes the cellular activator of the protein kinase, PKR. PACT and PKR are upstream components of a number of signal transduction and gene transcription pathways used by pro-inflammatory cytokines. We have investigated the role of PACT and PKR in articular cartilage degradation using cytokine treatment of bovine primary chondrocytes and cartilage explants. Tumour necrosis factor α increased expression of PACT protein after 3 h of treatment. Furthermore, increased phosphorylation of PKR and eukaryotic initiation factor 2-α was observed. The known role of PKR in cytokine-induced signalling pathways, together with our data showing cytokine regulation of PACT and PKR in chondrocytes, reveals a novel mechanism of cartilage degradation that may be important in the pathogenesis of arthritic diseases.

Introduction
Our previous studies [1] analysing the genes regulated in cartilage at the onset of spontaneous osteoarthritis (OA) in the guinea pig knee revealed up-regulation of the gene for protein kinase R (PKR)-activating protein (PACT), which encodes the cellular activator of the serine/threonine protein kinase, PKR [2]. PACT and PKR are upstream components of a number of signal transduction and gene transcription pathways including induction of the early response genes c-myc and c-fos [3], control of cellular proliferation [4] and apoptosis [5], pathways known to be involved in arthritic disease. Both PACT and PKR have been shown to induce apoptosis via activation of eukaryotic initiation factor 2-α (eIF2-α) and increased caspase activation [7,8]. Interestingly, although PACT and PKR have not previously been investigated in chondrocytes, several studies have implicated PKR in tumour necrosis factor α (TNF-α)-responsive signalling cascades in a number of other cell types [5,9,10]. Since pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α are strongly implicated in the pathogenesis of OA and rheumatoid arthritis (RA) (for a review see [11]), this suggests a novel role for this kinase in cartilage degeneration.

In the current study, we have used cytokine treatment of bovine primary chondrocytes and cartilage explants to investigate the role of PACT in TNF-α signalling.

Materials and methods
All materials were purchased from Sigma unless otherwise stated. Recombinant human cytokines were purchased from Peprotech EC.

References