Biochem. J. (1988) 250, 111–115 (Printed in Great Britain)

Effect of recombinant cytokines on glycolysis and fructose 2,6-bisphosphate in rheumatoid synovial cells *in vitro*

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Recombinant-derived human interleukin 1 (IL1) α and β and interferon γ (IFN-γ) each produced similar increases in rheumatoid synovial cell (RSC) glycolysis, as judged by increased values for glucose uptake, lactate production and cellular fructose 2,6-bisphosphate [Fru(2,6)P_2]. Measurement of Fru(2,6)P_2 proved to be the most sensitive parameter for an assessment of glycolysis: IL1α, IL1β and IFN-γ all produced a 3–6-fold increase in this metabolite whereas tumour necrosis factor (TNFα) was far less effective. Prostaglandin E production was stimulated predominantly by IL1α and IL1β rather than by IFN-γ or TNFα. When combinations of cytokines were examined the addition of IFN-γ with either IL1α, IL1β or murine IL1 produced a synergistic increase in cellular Fru(2,6)P_2. The three forms of IL1 increased Fru(2,6)P_2 via the same pathway, whereas IFN-γ acted via a different mechanism. The increase in Fru(2,6)P_2 in subcultured RSC produced by addition of medium from a primary culture exceeded the maximal effects of any of the single cytokines studied, suggesting the presence of a mixture of cytokines in the primary RSC culture medium.

INTRODUCTION

It has been known for many years that the glucose uptake and lactate production of rheumatoid synovial tissue is increased over that of normal [1]. In 1974 Castor [2] described a 'connective tissue activating peptide' prepared from human spleen which on addition to cultured normal synovial cells induced metabolic hyperactivity characterized by increased glucose uptake and lactate production. This effect is likely to have been the result of one or more of the increasing number of recently recognized cytokines now known to be present in rheumatoid synovial fluid [3–5]. Studies *in vitro* have revealed that three such factors, TNFα, IL1 and IFN-γ, suppress the cellular activity of lipoprotein lipase [6–8], one of the key enzymes of plasma triacylglycerol metabolism, but as yet little is known regarding their effects on carbohydrate metabolism. In view of the increased glycolysis reported for cells of the rheumatoid joint [9] we have examined the effects of these cytokines on the glycolytic and prostanoid metabolism of cultivated rheumatoid synovial fibroblasts. In addition to measurements of glucose uptake and lactate production to assess changes in glycolysis we have also measured intracellular Fru(2,6)P_2, which is known to be a powerful stimulator of 6-phosphofructo-1-kinase (EC 2.7.1.11), a regulatory enzyme of glycolysis [10] and which has been proposed as part of the mechanism by which mitogens increase glycolysis in cultured chick embryo fibroblasts [11]. We report here the ability of specific recombinant cytokines to modulate the glycolytic activity and PGE production of rheumatoid synovial cells *in vitro*.

MATERIALS AND METHODS

Cytokines

Recombinant human IL1α and recombinant murine IL1 were produced in *Escherichia coli* using recombinant DNA technology and had specific activities of 4×10^7 units/mg and 5×10^6 units/mg respectively in the thymocyte co-stimulation assay [12]. Recombinant IFN-γ had a specific activity of 3.7×10^6 units/mg based on antiviral activity. Recombinant TNFα, produced in *E. coli* by Genentech Inc. (San Francisco, CA, U.S.A.), had a specific activity of 5×10^7 units/mg in the murine L929 cell cytotoxicity assay [13] and was a generous gift from Boehringer Ingelheim, Vienna, Austria. Recombinant human IL1β was obtained from Cistron Technology, Pine Brook, NJ, U.S.A.; 1 unit of activity causes half-maximal incorporation of [^{3}H]thymidine in the thymocyte co-stimulation assay. Medium derived from primary cultures of adherent rheumatoid synovial cells (conditioned medium) provided a natural source of cytokine activity, described as synovial factor (SF). Such cultures contained a mixed population of synovial cells, predominantly of fibroblastic and macrophagic morphology.

Experimental protocol

Rheumatoid synovial tissue was dissociated enzymically and the cells cultured as previously described [14,15] in 6- or 12-well plates. Subconfluent monolayers of RSC were incubated at 37 °C in a water-saturated atmosphere of 5 % CO_2 in air, with and without cytokines

Abbreviations used: Fru(2,6)P_2, fructose 2,6-bisphosphate; IL1, interleukin 1; IFN-γ, interferon-γ; TNF, tumour necrosis factor; PGE, prostaglandin E; RSC, rheumatoid synovial cells; DMEM, Dulbecco's modified Eagle medium, SF, synovial factor; FCS, foetal-calf serum; HBSS, Hanks' balanced salt solution.

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Table 1. Effect of cytokines on RSC glycolysis and PGE production

Subcultured RSC were incubated with DMEM/FCS ± cytokine(s) for 21 h. The medium was removed and the cells washed with HBSS before adding DMEM for 30 min. After the second incubation the medium was removed and the cells processed for Fru(2,6)P_2 measurement as described in the Materials and methods section. Values are means ± S.E.M. ($n = 3$). All levels in treated cultures were significantly higher than control ($P < 0.05$)

Treatment	Glucose uptake (μmol/well)	Lactate production (nmol/well)	Cellular Fru(2,6)P_2 (pmol/well)	PGE production (ng/well)
DMEM/FCS	2.31±0.11	78±4	16±2	< 0.55
IL1α (5 units/ml)	3.22±0.11	113±6	82±6	149.1±1.2
TNFα (500 units/ml)	2.86±0.09	97±5	37±2	6.8±0.4
IFN-γ (200 units/ml)	3.22±0.07	119±2	102±4	3.1±0.1
IL1α+TNFα	2.83±0.02	98±1	102±5	148.9±6.8
IL1α+IFN-γ	3.37±0.09	118±3	254±11	152.0±5.2
TNFα+IFN-γ	2.92±0.10	101±2	55±2	16.4±1.3
TNFα+IL1α+IFN-γ	3.53±0.15	126±4	306±9	172.5±8.2

in DMEM supplemented with 10% FCS. Culture medium was collected after 18–24 h and stored at −20 °C until assayed for glucose utilization (40–60% of that originally present) and PGE production. The cells were washed several times with HBSS before adding DMEM for a further 30 min, after which the medium was removed and stored at −20 °C until assayed for lactate production. A second incubation in DMEM alone was required to assess lactate production since the initial incubation was performed in the presence of lactate-containing FCS. The cells were then either solubilized with 50 mM-NaOH/1% (v/v) Triton X-100 for Fru(2,6)P_2 measurement [11] or precipitated with 6% (w/v) $HClO_4$ for 'Lowry' protein [16]. The six cell preparations used in these experiments had been in culture for between 2 and 52 weeks and all gave qualitatively reproducible responses to the cytokines studied. Although the total cell protein content per well showed variation with different cell cultures (73–129 μg of protein/well), within a single experiment the variation in cell protein/culture well was consistently less than 5%.

Analytical procedures

Lactate production and glucose utilization by RSC were measured in the culture medium using standard enzymic techniques [17,18]. For measurement of Fru(2,6)P_2 the solubilized cells were scraped into tubes and heated for 10 min at 80 °C to denature protein and to stabilize Fru(2,6)P_2. After cooling in an ice–water bath the sample was made 20 mM in Hepes, adjusted to pH 7 with 1 M-acetic acid and centrifuged at 9000 g for 2 min. Fru(2,6)P_2 was assayed in the supernatant by the method of Van Schaftingen *et al.* [19] as modified by Van Schaftingen & Hers [20]. PGE was measured by conventional radioimmunoassay using an antiserum with similar specificity towards PGE_1 and PGE_2, as previously described [21].

RESULTS

Estimation of the rate of RSC glycolysis

In these experiments the three parameters used to estimate the likely flux through glycolysis produced similar data. For example, the cellular level of Fru(2,6)P_2 and rate of lactate production had a correlation coefficient of 0.72, whilst Fru(2,6)P_2 and glucose uptake had a correlation coefficient of 0.75 (data from Table 1); a dose–response study showed that the lowest concentration of IL1 significantly to increase Fru(2,6)P_2 and glucose uptake was the same (result not shown). Measurement of Fru(2,6)P_2 levels appeared to be the most sensitive indicator of the glycolytic rate, since the incremental responses were larger than those in lactate and glucose (Table 1). Experiments with RSC at various densities showed the effect of IL1 on glycolysis was less marked when the cells were confluent (result not shown), and for this reason all experiments were performed at sub-confluence.

Cytokines acting singly

The five cytokines all stimulated glycolysis in RSC as indicated by increased glucose uptake, lactate production and Fru(2,6)P_2 levels. IL1α and β and IFN-γ stimulated RSC glycolysis to a similar extent, causing a 3–6-fold increase in Fru(2,6)P_2, in contrast to TNFα which, even at a much higher concentration, resulted in only a small increase (Table 1 and Fig. 1*a*). Similarly, IL1α was a more potent stimulus than TNFα of RSC PGE production, a property ascribable to both cytokines. IFN-γ produced a dose-related increase in Fru(2,6)P_2 which was significantly greater than control at 1 unit/ml and maximal at 100 units/ml (Fig. 2). (Human) IL1α and β at 1 unit/ml produced the same maximal increase in Fru(2,6)P_2 and both were substantially more potent than the murine form (Fig. 1*a*). The effects of these three forms of IL1 on Fru(2,6)P_2 mirrored exactly the pattern of another IL1-induced response, namely PGE production (Fig. 1*b*). Inhibition of PGE production with piroxicam (14 μM) or indomethacin (14 μM) or the glucocorticoid dexamethasone (1 μM) did not prevent the IL1-induced increase in Fru(2,6)P_2, glucose uptake or lactate production (result not shown). However, the protein synthesis inhibitor cycloheximide (10 μg/ml) prevented the IL1-induced rise in Fru(2,6)P_2 (result not shown).

Cytokines in combination

The combined action of IL1α or β with IFN-γ caused an increase in RSC Fru(2,6)P_2 suggestive of synergism

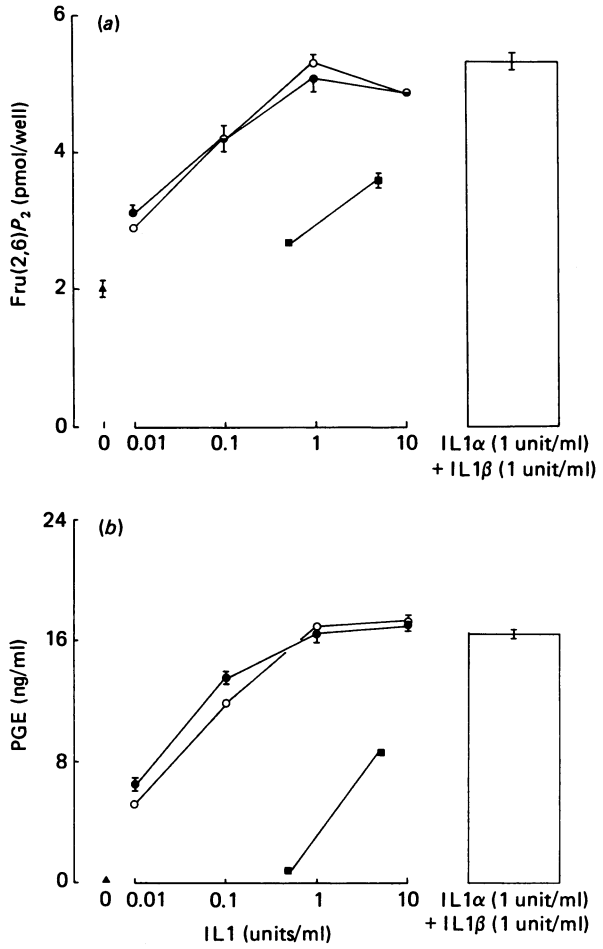

Fig. 1. Effect of increasing concentrations of IL1α and β and murine IL1 on RSC Fru(2,6)P_2 and prostaglandin E production

Subcultured RSC were incubated with DMEM/FCS (▲), IL1α (○), IL1β (●) or murine IL1 (■) for 23 h. The medium was removed for PGE measurement (*b*) and the cells processed for Fru(2,6)P_2 (*a*) measurement as described in the Materials and methods section. Values are means ± S.E.M. ($n = 3$).

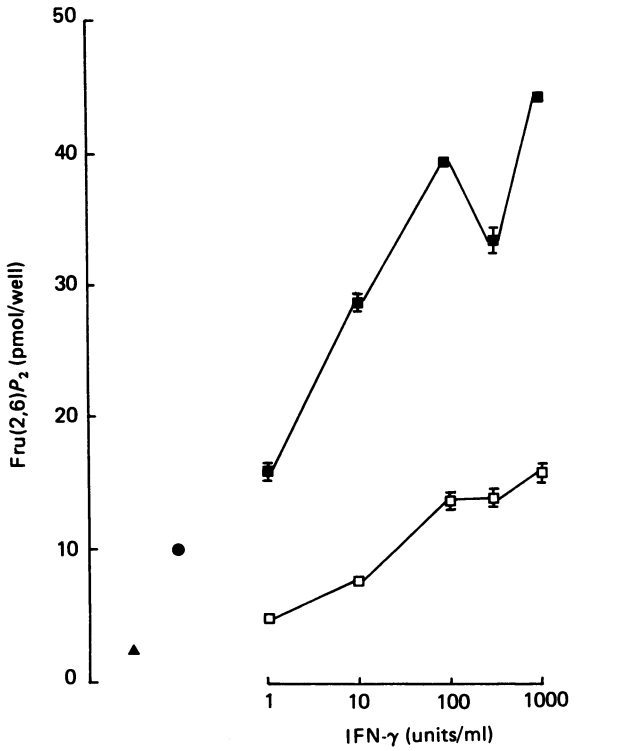

Fig. 2. Effect of increasing concentrations of IFN-γ with and without IL1 on RSC Fru(2,6)P_2

Subcultured RSC were incubated with DMEM/FCS (▲), 1 unit of IL1α/ml (●), IFN-γ (□) or 1 unit of IL1α/ml + IFN-γ (■) for 21 h. The medium was removed and the cells processed for Fru(2,6)P_2 measurement as described in the Materials and methods section. Values are means ± S.E.M. ($n = 3$).

Table 2. Comparison of both forms of IL1 on RSC Fru(2,6)P_2

Subcultured RSC were incubated with DMEM/FCS ± cytokine(s) for 24 h. The medium was removed and the cells processed for Fru(2,6)P_2 measurement as described in the Materials and methods section. Values are means ± S.E.M. ($n = 3$). All levels in treated cultures were significantly higher than control ($P < 0.01$).

Treatment	Cellular Fru(2,6)P_2 (pmol/well)
DMEM/FCS	1.7 ± 0.2
IL1α (1 unit/ml)	7.1 ± 0.8
IL1β (1 unit/ml)	7.9 ± 0.8
IFN-γ (300 units/ml)	7.1 ± 0.6
IL1α + IL1β	8.4 ± 0
IL1α + IFN-γ	27.6 ± 0.6
IL1β + IFN-γ	30.2 ± 1.3

between these two cytokines (Tables 1 and 2). This effect could be demonstrated over a wide range of IFN-γ concentrations, including those which without added IL1α produced only a trivial increase in Fru(2,6)P_2 (Fig. 2). A synergistic response was also observed when murine IL1 was combined with IFN-γ (result not shown). The combined addition of IL1α and IL1β at concentrations optimal for the stimulation of Fru(2,6)P_2 produced no additive effect even in the presence of added murine IL1 (Fig. 1*a* and Table 2).

The effect of combining TNFα with either IL1α or IFN-γ on RSC Fru(2,6)P_2 levels were not marked, and TNFα showed no evidence of any synergism with the other cytokines (Table 1). However, the combination of all three cytokines produced the highest values for both PGE production and the measured glycolytic parameters (Table 1).

In previous experiments we had confirmed that conditioned medium obtained from primary cultures of RSC, known as synovial factors (SF), stimulated PGE production by both synoviocytes and articular chondrocytes [21]. The addition of 5% (v/v) SF to RSC increased Fru(2,6)P_2 more than the maximal effects of IL1 or IFN-γ alone, and when combined with either cytokine the resulting increase was not synergistic (Table 3). The addition of SF with both IL1α and IFN-γ produced even

Table 3. Comparison of SF and cytokine effects on RSC Fru(2,6)P_2

Subcultured RSC were incubated with DMEM/FCS ± cytokine(s) or SF for 21 h. The medium was removed and the cells processed for Fru(2,6)P_2 measurement as described in the Materials and methods section. Values are means ± S.E.M. ($n = 3$). All levels in treated cells were significantly higher than control ($P < 0.001$).

Treatment	Cellular Fru(2,6)P_2 (pmol/well)
DMEM/FCS	5±1
IL1α (10 units/ml)	23±2
IFN-γ (300 units/ml)	19±1
SF (5%, v/v)	34±1
IL1α+SF	54±2
IFN-γ+SF	47±2
IL1α+IFN-γ	51±2
IL1α+IFN-γ+SF	69±1

higher Fru(2,6)P_2 levels than those observed when the two cytokines were present at concentrations producing maximal Fru(2,6)P_2 stimulation (Table 3).

DISCUSSION

The four human and one murine recombinant cytokines all stimulated RSC glycolysis as indicated by increases in glucose uptake, lactate production and, perhaps more significantly cellular Fru(2,6)P_2. Human IL1α and β and IFN-γ produced similar stimulation of RSC glycolysis and both were far more effective than TNFα. Human IL1α and β also produced a greater rise in cellular Fru(2,6)P_2 than did a higher concentration of the murine form. The greater potency of human IL1α and β over TNFα was paralleled by their relative ability to stimulate PGE production by these cells. Furthermore, in a dose-response study, 1 unit of either IL1α or β/ml produced maximal increases in both Fru(2,6)P_2 and PGE production, suggesting a possible relationship between the rate of glycolysis and PGE production. However, these two processes could be dissociated by non-steroidal anti-inflammatory substances such as piroxicam and indomethacin, or by a glucocorticoid, all of which eliminated the IL1-induced rise in PGE production without preventing the stimulation of glycolysis. Only by the inhibition of protein synthesis with cycloheximide was it possible to prevent the IL1-induced increase in glycolysis.

The observation that IFN-γ caused only a small increase in PGE production agrees with previous studies [22]. However, the finding that human IL1s were far more potent than TNFα differs from the results of Dayer *et al.* [23] who found that purified murine TNFα was more effective than murine recombinant IL1 at increasing RSC PGE production. Such differences may be partly explained by the use of human recombinant cytokines in the present experiments, especially as human IL1 was found to be more potent than murine IL1 in stimulating PGE production on a per unit basis.

Fru(2,6)P_2, synthesized by the enzyme 6-phosphofructo-2-kinase (PFK-2), is a potent stimulator of liver 6-phosphofructo-1-kinase (PFK-1), a regulatory enzyme of glycolysis [10]. Confirmatory studies on chick embryo fibroblasts showed that mitogenic doses of insulin or phorbol esters increased both Fru(2,6)P_2 concentrations and the glycolytic rate of those cells [11]. This study has confirmed that changes in cellular Fru(2,6)P_2 were correlated closely with cytokine-induced changes in glucose utilization and lactate production. The mechanism whereby these cytokines stimulate glycolysis via 6-phosphofructo-2-kinase activity is uncertain, but some information may be derived from the present data. The addition of each of the forms of IL1 with IFN-γ consistently gave a synergistic increase in Fru(2,6)P_2, suggesting that these two cytokines acted by a different mechanism and/or pathway. When two forms of IL1 were added together at optimal concentrations no additive effect was observed, which suggested that all types of IL1 act via the same pathway. This agrees with radioligand binding studies which reported that human IL1α and β compete for the same receptor site on both murine and human cells [24]. Since the increase in Fru(2,6)P_2 produced by TNFα alone was relatively small, it was not possible to establish whether the TNFα effect was mediated via the IL1, IFN-γ or another pathway.

Support for the proposal that these cytokines may be influencing glycolysis in the rheumatoid joint was provided by the finding that SF (conditioned medium removed from a primary RSC culture) increased cellular Fru(2,6)P_2. SF was previously used as a crude source of IL1 to stimulate PGE production in articular chondrocytes and synoviocytes [21] and from our results with recombinant IL1 it would therefore be expected to increase Fru(2,6)P_2. However, the addition of SF together with IFN-γ did not result in the expected synergistic rise in Fru(2,6)P_2 although a significant increase was achieved, an observation which suggests that the SF contained factors other than IL1 such as IFN-γ or an agent that acted via the IFN-γ mechanism. Furthermore, as the addition of SF to optimal concentrations of both IL1α and IFN-γ produced an even higher value for Fru(2,6)P_2 than the two cytokines together, this would suggest that SF contains another factor which affects Fru(2,6)P_2 levels.

Since increased glycolysis is a prerequisite for mitogen-stimulated fibroblast proliferation, the stimulation of glycolysis by IL1, IFN-γ and TNFα may be a feature of the mitogenic action of these cytokines on fibroblasts [25–27]. If this is so our measurements of Fru(2,6)P_2 suggest that IL1 is a far more potent mitogen for synovial fibroblasts than is TNFα, and that the combined presence of both IL1 and IFN-γ might further enhance proliferation, although this has yet to be examined experimentally.

These results provide evidence *in vitro* that IL1, TNFα and IFN-γ can affect not only lipid but also carbohydrate and prostanoid metabolism of fibroblastic cells. Modulation of cellular metabolism by these cytokines *in vivo* is indicated by the finding that cancer patients receiving recombinant IFN-γ show marked increases in plasma triacylglycerol levels and a decrease in plasma post-heparin lipase activity [28], and by the development of hyperglycaemia following injection of TNFα into rats [29].

Since IL1, IFN-γ and TNFα have recently been detected and measured in rheumatoid synovial fluids [3–5], the

enhanced glycolysis found in synovial cells from a rheumatoid joint may well be due to the action of one or more of these cytokines. The present findings that IL1 stimulates glycolysis and PGE production over a concentration range which affects other cellular processes such as thymocyte proliferation, whereas very high levels of activity of TNFα are required to produce even small changes, suggest a primary role for the forms of interleukin I in these processes.

This work was supported by the Arthritis and Rheumatism Council and the North West Regional Health Authority. We thank Mrs A. Elliott and Mrs M. Williamson for preparation of the manuscript.

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Received 15 June 1987/30 August 1987; accepted 15 October 1987