Spontaneous and cytokine regulated *c-fos* gene expression in rheumatoid synovial cells: resistance to cytokine stimulation when the *c-fos* gene is overexpressed

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Abstract

Objective—To study the effect of cytokines on the transactivation of the *c-fos* gene in relation to the contribution of overexpression of *c-fos/AP-1* in rheumatoid joint destruction.

Methods—The promoter region (−447 to +109) of the human *c-fos* gene was integrated upstream of the chloramphenicol acetyltransferase (CAT) reporter gene, and the effect of cytokines on the expression of the *c-fos* gene was studied in the rheumatoid synovial cells of early (3–4) or late (14–18) passages, in the presence or absence of cytokines, by the transient transfection assay.

Results—Expression of *c-fos* gene was enhanced by tumour necrosis factor α (TNF α) and interleukin 6 (IL6) in the synovial cells of early passage, whereas it was not enhanced in the synovial cells of late passage. The *c-fos* gene expression was also enhanced by 13-*O*-tetradecanoyl phorbol-13-acetate (TPA) in early passage but was somewhat suppressed in the late passage. It was found that the *c-fos* gene and c-Fos protein were both increased in the synovial cells of late passage. Similarly, *c-fos* gene expression was also not increased by TPA or cytokine stimulation in the stable *c-fos* transformants (fos-ph8) or H-ras transformed NIH3T3 cells (NIH H-ras cells) that constitutively expressed *c-fos* genes.

Conclusions—Although TNF α and IL6 augmented *c-fos* gene expression of rheumatoid synovial cells, transactivation of *c-fos* gene became resistant against cytokine stimulation under prolonged expression of *c-fos* gene, which may impart a tumour-like characteristic to rheumatoid synovial cells.

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Rheumatoid arthritis (RA) is a chronic polyarthritis of unknown cause characterised by irreversible joint destruction.¹ Previous studies in this laboratory have shown that characteristics of rheumatoid joint destruction, synovial overgrowth, and osteoporosis, especially of the juxta-articular region, are experimentally produced by augmenting *c-fos* gene expression.^{2–6}

As the *c-fos* gene has been shown to be augmented in rheumatoid joints,^{7,8} we studied the

role of inflammatory cytokines in the transactivation of the *c-fos* gene to investigate the nature of rheumatoid synovial cells in relation to overexpression of the *c-fos* gene. Human *c-fos* gene expression was studied in rheumatoid synovial cells by transient transfection assay in the presence or absence of cytokines. The results indicated that *c-fos* gene expression was augmented by tumour necrosis factor α (TNF α) and interleukin-6 (IL6) in the rheumatoid synovial cells of early passage, whereas those of late passage became resistant to cytokine stimulation. This is discussed in relation to a tumour-like overgrowth of synovial cells in RA.

Patients and methods

REAGENTS

12-*O*-tetradecanoyl phorbol-13-acetate (TPA) was purchased from Sigma Chemical Co, MO. TNF α and interferon γ (IFN γ) were obtained from Hayashibara Biochemical Co Ltd, Okayama, Japan. Interleukin 1 β (IL1 β) was obtained from Otsuka Pharmaceutical Factory Inc, Tokushima, Japan. Interleukin 2 (IL2) was purchased from Boehringer Mannheim GmbH, Mannheim, Germany. Recombinant IL6 and TNF α were purchased from R & D Systems, Inc (Minneapolis, MN). Rat anti-human IL6 monoclonal antibody (mAb) was purchased from Pharmingen Co Ltd San Diego, CA), and goat antihuman TNF α mAb was obtained from Genzyme Techne Corp (Cambridge, MA).

SYNOVIAL CELL CULTURE

Synovial tissues were obtained during joint surgery from patients with RA fulfilling the diagnostic criteria of the American College of Rheumatology⁹ and from patients with osteoarthritis (OA). Tissues were treated with 2 mg/ml of *Clostridium histolyticum* collagenase (Wako Pure Chemicals, Osaka, Japan) at 37°C for 45 minutes, followed by treatment with 0.05% trypsin (Difco Laboratories, Detroit, Michigan, USA) at 37°C for 30 minutes.¹⁰ Dispersed synovial cells were placed in a 10 cm tissue culture dish (Iwaki Glass Co Ltd, Tokyo, Japan), and cultured overnight in Iscove's modified Dulbecco's minimal essential medium (IMDM; Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS; Whittaker MA Bioproducts Inc, Walkersville, MD). Adherent synovial cells were harvested and passaged into another dish. Harvest and passage were repeated twice a week. For the

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Figure 1 Human c-fos promoter (from -447 to +109) was integrated into pBL-CAT2 plasmid to construct the CAT reporter gene (pBL-fos-CAT). Binding sites on DNA and transcription factors were illustrated: SIF = sis (platelet derived growth factor)-induced factor; Elk-1 = 62 kDa ternary complex factor; SRF = serum response factor-SRE = serum response element; AP-1 = activator protein 1; TRE = 12-O-tetradecanoyl phorbol-13-acetate (TPA) response element; NFI-like = nuclear factor I-like binding activity; DRBP = GC-rich direct repeats binding protein; CREB = cAMP response element binding protein; CRE = cAMP response element; TATA = TATA box.

transfection study, synovial cells of early passage (3–4 passage) and late passage (14–18 passage) were used. Anti-IL6 mAb (0.1 µg/ml) and anti-TNFα mAb (0.04 µg/ml) were added every four days.

DNA CONSTRUCT

The promoter region (from -447 to +109) of the human c-fos gene was amplified from genomic DNA by the polymerase chain reaction.^{11 12} For the chloramphenicol acetyl-transferase (CAT) enzyme linked immunosorbent assay (ELISA), the promoter was integrated into the *Hind* III and *Bam*HI sites of the pBL-CAT2 bacterial CAT plasmid¹³ in order to locate at the upstream position of the CAT reporter gene (pBL-fos-CAT) (fig 1).

CAT ELISA ASSAY

Synovial cells were plated at 0.5×10⁵ cells/2 ml in a 3.5 cm dish. When the cells became subconfluent, 1 µg of DNA (pBL-fos-CAT) was transfected using lipofectin (Lipofect A; Gibco BRL, Tokyo, Japan). One day later, cells were treated with a panel of cytokines and cultured for a further 24 hours. The amount of CAT in lysed cells was measured by a CAT ELISA (Boehringer Mannheim) using a microplate reader model 550 (Nippon Bio-Rad Laboratories, Tokyo, Japan).

WESTERN BLOT ANALYSIS

Cells (5×10⁶) were suspended in 100 µl of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM

NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF). After sonication using Microson (MS50, Heat Systems Ultrasonics, Inc, Farmingdale, NY), the soluble fraction was obtained by centrifugation at 12 000 rpm for 10 minutes. Fifty microlitres of protein was separated using 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore Corp, Bedford, MA). After blocking with 3% bovine serum albumin, the membrane was incubated with rabbit IgG anti-c-Fos antibody (sc-52; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for two hours. The membrane

Table 1 Rheumatoid synovial cells at early passage were stimulated with interferon γ (IFNγ), tumour necrosis factor α (TNFα), interleukin 1β (IL1β), interleukin 2 (IL2), or interleukin 6 (IL6) for 24 hours. They were assayed for c-fos expression in a transient assay using pBL-fos-CAT

Cytokine	10 U/ml	50 U/ml	100 U/ml
Exp. 1			
IFNγ	1.1*	1.0	1.3
TNFα	1.2	1.0	5.6
IL1β	1.2	1.3	1.2
IL2	1.1.	1.1	1.1
IL6	1.2	1.6	2.2
Medium	0.9		
Exp. 2			
IFNγ	1.0	2.0	0
TNFα	1.2	10.0	8.2
IL1β	2.2	1.6	1.8
IL2	1.6	0.2	2.0
IL6	1.2	3.0	6.2
Medium	1.2		

*pg/ml.

Figure 2 (A) Rheumatoid synovial cells at late passage were stimulated and assayed ($n=2$) as described in table 1. (B) Rheumatoid synovial cells at early passage and late passage were compared. Cells were cultured with or without $10 \mu\text{M}$ 12-O-tetradecanoyl phorbol-13-acetate (TPA) for 24 hours and assayed for chloramphenicol acetyltransferase (CAT) activity ($n=2$).

Figure 3 Western blot analyses of *c-Fos* protein in the cultured synovial cells of rheumatoid arthritis (RA) and osteoarthritis (OA).

was incubated with horseradish peroxidase-conjugated antirabbit IgG antibody (Amersham International, Buckinghamshire, England), and bound antibody was visualised using an enhanced chemiluminescence system (ECLTM; Amersham).

FO5-PH8 TRANSFORMANT

Cultured synovial cells (1×10^6) were suspended in 200 μl of Dulbecco's phosphate buffer without calcium (Nissui Pharm. Co Ltd, Tokyo, Japan) containing 20 μg of pH 8 or fos-PH8 vectors.³ After incubation on ice for 10 minutes, electroporation was carried out five times (five pulses) in a 2 mm gap cuvette at 700 V for 99 μs using an Electro Square Porator T820 (BTX Inc, San Diego, CA). Cells were then cultured in IMDM containing 10% FBS before selection of stable transformants by G418. Stable fos-PH8 transformants were subsequently transfected by pBL-fos-CAT for CAT assay.

NIH H-RAS CELL

H-ras transformed NIH3T3 cells were maintained in IMDM,¹⁴ and used for transfection experiments.

Figure 4 Western blot analyses of *c-Fos* protein. (A) Synovial cells of patients with osteoarthritis (OA) were cultured with recombinant IL6 and TNF α for three weeks. (B) Rheumatoid synovial cells were cultured with anti-IL6 and anti-TNF α monoclonal antibody for three weeks.

STATISTICAL ANALYSIS

The data were expressed as means (standard deviation). Statistical analyses were performed using the Student's *t* test.

Results

When synovial cells of early passage were stimulated with a panel of cytokines for 24 hours in a transient transfection assay using pBL-fos-CAT reporter, expression of the *c-fos* gene was enhanced by TNF α and IL6 (table 1). However, the *c-fos* gene was spontaneously expressed before stimulation with cytokines when synovial cells of late passage were used (fig 2A). Synovial cells of late passage did not respond to cytokines such as IL6 or TNF α (fig 2A) or to cytokines such as IFN γ , IL1 β , or IL2 (not shown). The *c-fos* gene expression in the synovial cells of late passage was somewhat inhibited by TPA (fig 2B).

Western blot analyses showed that c-Fos protein levels were significantly increased in the rheumatoid synovial cells of late passage as compared with OA, whereas the increase of c-Fos was less significant in the rheumatoid synovial cells of early passage (fig 3). In contrast, the c-Fos protein was decreased in the synovial cells of patients with OA at late passage, suggesting that spontaneous increase of c-Fos and its transcriptional activity was specific for RA. To test this possibility further, synovial cells of patients with OA were cultured in the presence of recombinant IL6 and TNF α for three weeks. The level of c-Fos protein was, however, not increased by these cytokines (fig 4). It was also found that the level of c-Fos protein was not decreased in the rheumatoid synovial cells cultured with anti-IL6 and anti-TNF α mAb for three weeks (fig 4).

Contribution of the increased expression of the *c-fos* gene was next studied in a stable transformant containing the fos-PH8 vector. The fos-PH8 transformant overexpressing the *c-fos* gene showed increased transcriptional activity through the human *c-fos* promoter, and transactivation of the *c-fos* gene was somewhat inhibited by TPA which was compatible with fig 2B (fig 5A). This was not the case for the control transformants containing the pH8 vector alone (fig 5B). Expression of the *c-fos* gene was also studied in H-ras transformed NIH3T3 cells (NIH H-ras cells). Expression of the *c-fos* gene in the NIH H-ras cells was higher

Figure 5 (A) Synovial cell transformants stably transfected with the *fos*-PH8 vector (*fos*-PH8) were stimulated with 10 μ M 12-O-tetradecanoyl phorbol-13-acetate (TPA). Expression of the *c-fos* gene was assessed by a chloramphenicol acetyltransferase (CAT) assay using pBL-*fos*-CAT ($n=2$). (B) Stable transformants containing the PH8 vector were assayed in an identical fashion to (A) ($n=2$). (C) NIH H-ras cells overexpressing the *c-fos* gene were stimulated by cytokines for 24 hours. TPA was used at 10 μ mol/l. Expression of the *c-fos* gene was assayed in an identical fashion to (A) ($n=2$).

because of H-ras activation.¹⁴ We found that transcriptional activation of *c-fos* CAT was increased in these cells (fig 5C). These cells also did not respond to stimulation with cytokines, except for a slight but insignificant increase by IL6 (fig 5C).

Discussion

Increased *c-fos*/AP-1 may contribute significantly to the pathogenesis of rheumatoid synovial overgrowth and joint destruction.¹⁻⁸ As previous studies have shown that cytokines such as IFN γ , TNF α , IL1 β , IL2, or IL6 increased the expression of *c-fos*/AP-1,¹⁵⁻¹⁹ it is likely that these cytokines stimulate transactivation of *c-fos*/AP-1 in inflamed rheumatoid joints. Nevertheless, because this has not yet been proved, we originally undertook this study to establish the relative order of contribution of various cytokines to the transactivation of *c-fos*/AP-1 in the rheumatoid synovium. However, instead we found that the cells overexpressing the *c-fos* gene seemed to behave differently and they did not respond normally to cytokine stimulation.

We, therefore, tested the effect of various cytokines on the transactivation of the *c-fos* gene in a transient transfection assay using a human *c-fos* gene promoter integrated upstream of the CAT reporter gene. The results showed that TNF α and IL6 augmented *c-fos* gene expression in rheumatoid synovial cells of early passage, indicating that these cytokines

should primarily be responsible for the transactivation of *c-fos*/AP-1 in rheumatoid synovium. The result is consistent with previous reports of the role of TNF α or IL6 in rheumatoid joint destruction.²⁰

We have also found that rheumatoid synovial cells of late passage acquire the ability to transactivate the *c-fos* gene spontaneously without prior stimulation by cytokines. The cells did not respond to stimulation with inflammatory cytokines such as TNF α and IL6. Therefore, it was possible that *c-fos* gene expression had already reached a plateau in these rheumatoid synovial cells of late passage and thus they did not respond further to exogenous cytokines. Spontaneous increase of *c-fos* expression and c-Fos protein in rheumatoid synovial cells after long culture seemed to be specific for RA as a similar increase was not found in the synovial cells of OA. The reason for this increase is at present unclear. However, as increased levels of the c-Fos protein in rheumatoid synovial cells were not reversed by the presence of antibodies against cytokines, they seemed to be intrinsic to long cultured rheumatoid synovial cells. This finding is consistent with that of Bucala *et al*, who found that the proliferative capacity of conditioned media from rheumatoid synovial cells continues for a long time but gradually declines at late passage.²¹

We next tested whether or not the inability of synovial cells cultured for a long time to respond to cytokines was due to overexpression

of the *c-fos* gene. We examined transcription of the *c-fos* gene in the fos-pH8 or NIH H-ras cells in which *c-fos* gene expression was increased. The result showed that transactivation of the *c-fos* gene was indeed increased in these cells and they did not respond to cytokine stimulation. It seems that synovial cells acquire some kind of resistance to cytokine stimulation, probably because of the increased *c-fos* gene expression. It seems interesting that, in the cells in which c-Fos was overexpressed, transcriptional activation of the *c-fos* gene by TPA was somewhat inhibited. As AP-1 sites should be occupied by the *c-fos*/AP-1 complex in the cells overexpressing the *c-fos* gene, signals for transactivating the *c-fos* gene could be rather inhibited, as was the case with glucocorticoid receptor binding on the AP-1 site.²² Therefore, overexpression of *c-fos*/AP-1 seems to impart a tumour-like characteristic to rheumatoid synovial cells.²

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