Interleukin-1 expression by neutrophils in rheumatoid arthritis

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Abstract

Objective—To determine if neutrophils from blood and synovial fluid of patients with rheumatoid arthritis and other joint arthropathies express interleukin-1 β mRNA.

Methods—RNA was isolated from neutrophils from patient and control blood, and synovial fluid of patients, probed in northern blots, and quantified by densitometry. It was also isolated and analysed from control blood neutrophils after incubation in vitro with granulocyte macrophage colony stimulating factor (GM-CSF).

Results—Neutrophils from the synovial fluid of patients with rheumatoid arthritis contained low levels of mRNA for interleukin-1 β —between 0.1 and 2% of those observed during stimulation of control neutrophils with GM-CSF for one hour. Higher levels (4–40% of the maximal GM-CSF values) were observed in blood neutrophils from patients with rheumatoid arthritis.

Conclusions—Neutrophils contribute to the cytokine network in rheumatoid arthritis. In some circumstances, activation of transcription may occur within the circulation of these patients.

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In rheumatoid arthritis, the normally acellular joint fluid is infiltrated by a variety of immune cells, including T and B lymphocytes, mononuclear phagocytes, and neutrophils.¹ During active disease, most of the infiltrating leucocytes are neutrophils and these have been activated in situ to release toxic reactive oxidants and granule enzymes.^{2,3} Rheumatoid synovial fluid contains many cytokines which may be produced by mononuclear phagocytes and endothelial cells. However, it is known also that neutrophils can secrete a variety of cytokines,^{4–8} the amounts secreted being generally between five and 10-fold less (on a cell basis) than levels produced by mononuclear phagocytes. Nevertheless, because neutrophils can infiltrate tissues in vast numbers, neutrophil derived cytokines are pathophysiologically important.

We have investigated whether neutrophils contribute to interleukin-1 β expression in rheumatoid disease. This cytokine is present in high concentrations within diseased joints and is implicated in disease pathology.^{9,10} It has been shown that a variety of proinflammatory mediators induce transient expression of this

cytokine in human neutrophils.^{8,11} A recent investigation of interleukin-1 β expression by rheumatoid neutrophils failed to detect ex vivo protein secretion in synovial cells, but the protein was detected in some blood neutrophils from rheumatoid patients.¹² We have measured interleukin-1 β mRNA in blood and synovial fluid neutrophils from patients with a variety of joint arthropathies.

Materials and methods

PATIENTS

We studied 26 patients attending the rheumatology clinics at the Royal Liverpool University Hospital, all with classical or seropositive rheumatoid arthritis (American Rheumatism Association criteria). Others examined were: two patients with seronegative polyarthritis, two with ankylosing spondylitis, one with Lyme's disease, and one with psoriatic arthritis. All were receiving non-steroidal anti-inflammatory drugs and none received steroids within the previous six months.

PREPARATION OF NEUTROPHILS

Neutrophils were isolated from buffy coats by sedimentation in dextran/Ficoll-Hypaque, as described previously.¹³ Neutrophils were also isolated from the blood and synovial fluid of patients with rheumatoid arthritis and other joint arthropathies as described previously.^{2,3} After purification, cells were suspended in RPMI 1640 medium and counted using a Fuchs-Rosenthal haemocytometer. Cell purity and viability were routinely assessed by Wright's staining and Trypan Blue exclusion and found to be >97% and >90%, respectively, for all preparations.

RNA EXTRACTION

Neutrophils from buffy coats were incubated at 10⁷ cells/ml in RPMI 1640 with or without 50 U/ml granulocyte macrophage colony stimulating factor (GM-CSF) at 37°C for periods of up to 24 hours. After incubation for 1, 2, 4 and 24 hours, samples were removed for isolation of RNA. RNA was also extracted from patient or control blood neutrophils and patient synovial fluid neutrophils immediately after isolation. Cell pellets were suspended in 4 mol/l guanidinium isothiocyanate, 5% β -mercaptoethanol, 50 mmol/l EDTA, 50 mmol/l Tris, pH 7.0, and lysed by being drawn through a 23 gauge needle to shear chromosomal DNA. The suspension was layered onto a caesium chloride (CsCl)/EDTA gradient (5.7 mol/l CsCl,

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50 mmol/l EDTA, density = 1.3995 ± 0.001) and centrifuged at 100 000 *g* for 16 hours at 14°C. The RNA pellet was suspended in diethylpyrocarbonate (DEPC) treated water and precipitated overnight with 0.3 mol/l sodium acetate and 2.5 vol ethanol at -80°C. The RNA was quantified by ultraviolet spectroscopy (typically 2 µg RNA/10⁷ neutrophils) and stored in 10 µg aliquots at -80°C as ethanol precipitates.

NORTHERN BLOTS

Aliquots of RNA (10 µg) were subjected to electrophoresis on 1.2% agarose gels (containing 1% (v/v) formaldehyde, 20 mmol/l 3-*N*-morpholinopropane sulphonic acid, 1 mmol/l EDTA, 5 mmol/l sodium acetate, pH 8.0) for 16 hours at 5 mA. The gels were then stained in ethidium bromide and RNA was visualised by ultraviolet illumination. The RNA was then transferred by capillary blotting onto Zetaprobe GT nylon membrane (Bio-Rad) in 20 × salt/sodium citrate (SSC) (3 mol/l NaCl, 0.3 mol/l sodium citrate, pH 7.0). Filters were baked at 80°C for 30 minutes, stored at room temperature in Saranwrap until use and then probed with labelled inserts prepared from the following cDNA clones obtained from the American Type Culture Collection (ATCC): interleukin-1β (ATCC 67024); v-fms (ATCC 41016); β-actin (ATCC 65128). The cDNA inserts were excised using appropriate restriction endonucleases and isolated from vector DNA by electrophoresis in low melting point agarose. Each insert (50–100 ng) was radiolabelled by random primed labelling using 25 µCi [³²P]-cytidine triphosphate (CTP) for 20 hours at 16°C. Unincorporated [³²P]-CTP was removed from the labelled cDNA using NucTrap columns (Stratagene). Radioactivity incorporated was typically > 10⁸ cpm/µg DNA. The filters were prehybridised for 30 minutes at 65°C in 0.25 mol/l Na₂HPO₄·7H₂O/7% sodium dodecyl sulphate (SDS), pH 7.2, and then hybridised with the probe for 16–20 hours in the same buffer at 65°C. Filters were washed in 20 mmol/l Na₂HPO₄·7H₂O/5% SDS, pH 7.2, twice for 30 minutes at 65°C, followed by two 10 minute washes in 20 mmol/l Na₂HPO₄·7H₂O/0.1% SDS at the same temperature. They were then blotted dry, wrapped in Saranwrap and exposed to x ray film (Fuji RX) for 24–48 hours. After autoradiography, probes were removed by heating the filters to 95°C in 0.1 × SSC containing 0.5% SDS, twice for 20 minutes. After autoradiographic confirmation of removal of the probe, the filters were reprobed.

MATERIALS

Dextran T500 and Ficoll-Hypaque were from Pharmacia (St Albans, UK), RPMI 1640 medium was from Flow Laboratories (Rickmansworth, UK), and the random labelling kit was from Boehringer (Lewes, UK). Zetaprobe was from Bio-Rad (Hemel Hempstead, UK), [³²P]-CTP from Amersham

(Little Chalfont, UK), and rGM-CSF was from Glaxo (Greenford, UK). The last was a non-glycosylated peptide with an activity of ≥ 1.5 mU/mg in the AML-193 proliferation assay. All other reagents were of the highest purity available.

Results

Addition of 50 U/ml GM-CSF to normal blood neutrophils for one hour induced a large increase in mRNA levels for interleukin-1β (fig 1A–C, lane 1). However, in mRNA of synovial fluid neutrophils from 21 patients with rheumatoid arthritis, transcripts for interleukin-1β were present at low, but detectable levels (fig 1A–C, lanes 2–7), being 0.1–2% of levels maximally induced by exposure of control blood neutrophils (from healthy donors) to GM-CSF *in vitro*. To obtain the results shown in this figure, the filters were exposed to film for seven days in order to visualise these low hybridisation signals. Similar levels of mRNA for interleukin-1β were detected in neutrophils from the synovial fluid of two patients with seronegative polyarthritis, but none was detected in neutrophils from two patients with ankylosing spondylitis, one with Lyme's disease or one with psoriatic arthritis (data not shown).

When the filters were stripped and probed for v-fms mRNA (the macrophage colony stimulating factor receptor), no hybridisation signals were detected (data not shown), thus

Figure 1 Interleukin-1β mRNA levels in control blood and patient synovial fluid neutrophils. **A–C:** Analysis of RNA from control blood neutrophils after incubation with GM-CSF for one hour *in vitro* (LANE 1) and neutrophils from the synovial fluid of patients with rheumatoid arthritis (LANES 2–7) (different controls and different patients). Filters exposed to film for seven days then stripped and probed for levels of actin mRNA. **D:** RNA isolated immediately after extraction of neutrophils from control venous blood (LANES 1–3), or from buffy coats (LANES 4–7). Transcript sizes: interleukin-1β 1 kb; actin 1.3 kb.

excluding the possibility that monocyte contamination of the neutrophil preparations was contributing to the interleukin-1 β signal. However, when the filters were reprobbed for actin mRNA, the hybridisation signals were equal to or greater than those obtained with control blood neutrophils after GM-CSF treatment *in vitro*.

Transcripts of mRNA for interleukin-1 β were detected in blood neutrophils of patients with rheumatoid arthritis at greater levels than those detected in paired synovial fluid neutrophils. Values for the blood neutrophils (expressed as a percentage of the maximal levels obtained after treatment of control blood neutrophils for one hour *in vitro* with GM-CSF) were: 42%, 23%, 20%, 16%, 5%, and 4%. Figure 2 shows northern blots of blood neutrophils from five patients, together with the paired synovial fluid neutrophil samples, from filters exposed to film for two days to develop the interleukin-1 β signal. In two patients with seronegative arthritis and two patients with ankylosing spondylitis, the hybridisation signals for interleukin-1 β mRNA in the blood neutrophils were undetectable (data not shown).

Discussion

Much evidence indicates that neutrophils contribute to the cytokine cascade during inflammation. Previous work has shown that mRNA for interleukin-1 β is not expressed in

blood neutrophils, but expression is increased rapidly upon exposure to agents such as GM-CSF. We could not detect transcripts for this cytokine in more than 20 preparations of neutrophils freshly isolated from venous blood or buffy coats of healthy controls (for example, figure 1D), confirming that interleukin-1 β is not expressed in blood neutrophils under normal circumstances.

Some reports indicate that synovial fluid neutrophils do not produce interleukin-1 β ,¹² whereas others have shown that they do.¹⁴ In addition, synovial fluid neutrophils express interleukin-8 and interleukin-1 receptor antagonist (interleukin-1ra).¹⁵⁻¹⁷ Expression of interleukin-1ra occurs in blood neutrophils and expression is upregulated by a variety of proinflammatory agents such as lipopolysaccharide, GM-CSF, and tumour necrosis factor. Levels of interleukin-1ra production by activated neutrophils can approach those produced by lipopolysaccharide treated monocytes.¹⁸ Thus synovial fluid neutrophils may exert both pro- and anti-inflammatory effects, depending upon local circumstances within the joint.

The results presented here indicate low, but detectable levels of mRNA for interleukin-1 β in synovial fluid neutrophils from patients with rheumatoid arthritis. In control neutrophils exposed to GM-CSF *in vitro*, levels of interleukin-1 β mRNA were maximal within one hour of stimulation and then declined rapidly to basal levels, being only about 10% of maximum four hours after stimulation and undetectable by 24 hours (data not shown). Low levels of mRNA for interleukin-1 β in a population of synovial fluid neutrophils may thus indicate heterogenous expression (only a small number of cells express), or may be a reflection of a post-stimulation value—that is, levels of mRNA are returning to a post-stimulatory value. It was therefore somewhat surprising to discover much higher levels of mRNA for interleukin-1 β in neutrophils isolated from the blood of rheumatoid arthritis patients. Levels of this transcript in some patients were comparable to those observed two hours after stimulation of control neutrophils with GM-CSF *in vitro*. Because of the very transient nature of increased mRNA levels, this observation indicates that blood neutrophils of these patients are continuously exposed to agents that stimulate transcription of interleukin-1 β . Previous work has shown that production of interleukin-1 β protein follows 4–18 hours after stimulation by GM-CSF.^{11, 18} Whether increased interleukin-1 β mRNA levels in blood neutrophils lead to systemic protein secretion, or to production once they are recruited into the joint, remains to be determined. Our results imply that activation of interleukin-1 β expression by neutrophils in rheumatoid disease can occur within the circulation *before* the cells enter diseased joints.

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Figure 2 Interleukin-1 β expression in blood and synovial fluid neutrophils from patients with rheumatoid arthritis. Patient RNA analysed and quantified on blots that also contained RNA from control blood neutrophils treated with GM-CSF *in vitro*. After probing for interleukin-1 β mRNA (filters exposed to film for two days), filters were stripped and probed for actin mRNA. **A:** LANE 1, control blood neutrophils; LANE 2, control blood neutrophils incubated for one hour with 50 U/ml GM-CSF; LANE 3, blood neutrophils from patient 1; LANE 4, synovial fluid neutrophils from patient 1; LANE 5, blood neutrophils from patient 2; LANE 6, synovial fluid neutrophils from patient 2; LANE 7, blood neutrophils from patient 3; LANE 8, synovial fluid neutrophils from patient 3. **B:** LANE 1, control blood neutrophils; LANE 2, control blood neutrophils incubated for one hour with 50 U/ml GM-CSF; LANE 3, blood neutrophils from patient 4; LANE 4, synovial fluid neutrophils from patient 4; LANE 5, control blood neutrophils; LANE 6, blood neutrophils from patient 5; LANE 7, synovial fluid neutrophils from patient 5.

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