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# Inflammatory cytokines induce synthesis and secretion of *gro* protein and a neutrophil chemotactic factor but not β2-microglobulin in human synovial cells and fibroblasts

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Exposure of human synovial cells and fibroblasts in monolayer culture to interleukin 1 results in prominent secretion of proteins with $M_r$ values of 6000 and 7000. By *N*-terminal sequence analysis, the $M_r$-6000 protein is identified as the protein encoded by a recently described *gro* mRNA. The $M_r$-7000 protein is identical to a neutrophil chemotactic factor released from monocytes. Stimulation of normal human fibroblasts with tumour necrosis factor α also results in expression and secretion of these two proteins. In addition to these cytokine-induced proteins, we have identified β2-microglublin as an $M_r$-8000 protein constitutively secreted by synovial cells.

## INTRODUCTION

Interleukin 1 (IL-1) is a pleiotropic mediator of the inflammatory response with diverse effects on several cell types [1]. In mesenchymal cells such as fibroblasts, synovial cells and chondrocytes, IL-1 induces expression and secretion of prostaglandin $E_2$, plasminogen activator, latent metalloproteinases and procollagens [1–9]. Expression of these molecules *in vivo* is probably essential for tissue remodelling and wound healing. Tumour necrosis factor α (TNFα) is also an important mediator of the inflammatory response with properties which overlap considerably with those of IL-1 [10].

We have been interested in characterizing proteins which are induced in connective tissue cells by these cytokines [6,7,11]. We report here that IL-1, and in some instances TNFα, induce expression and secretion of $M_r$-6000 and -7000 proteins from normal human fibroblasts and synovial cells. In additon to these two proteins, an $M_r$-8000 protein is constitutively secreted from synovial cells. These three proteins are identified here by *N*-terminal sequence analyses and their relationship to recently described proteins is discussed.

## EXPERIMENTAL

### Cell culture

Human synovial cells from the tissue of osteoarthritic and rheumatoid arthritic patients undergoing joint replacement were derived by proteolytic digestion [6] and used between the 4th and 17th passages. Human fetal lung fibroblasts (HFL-1; American Type Culture Collection, Rockville, MD, U.S.A.) and apparently normal human skin fibroblasts (AG4148, Genetic Mutant Cell Repository, Camden, NJ, U.S.A.) were used between the 12th and 17th passages [6]. Cells were plated at $5 \times 10^4$ cells/cm$^2$ in multi-well plates (Costar, Cambridge, MA, U.S.A.) in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal calf serum. After three days, the monolayers were rinsed extensively to remove serum and incubated in minimum essential medium (MEM) (methionine- or cysteine-free) (GIBCO, Grand Island, NY, U.S.A.) containing 30 μCi/ml of [$^{35}$S]methionine (NEG-009T, New England Nuclear) or [$^{35}$S]cysteine (NEG-022T) respectively. To prevent depletion of methionine or cysteine, the MEM was supplemented with non-radioactive methionine or cysteine respectively to a final concentration of $0.2 \times 10^{-4}$ M. Recombinant human α and β IL-1 ($10^7$ U/mg, Cistron, Pine Brook, NJ, U.S.A.) were added at 0.1–20 U/ml. Recombinant human TNFα (Genzyme) was added at 0.4–40 ng/ml. The amount of endotoxin present in the recombinant cytokines (maximum 0.2 pg/ml in final dilution) was insufficient to activate synovial cells and fibroblasts.

### SDS/polyacrylamide-gel electrophoresis (SDS/PAGE)

Upon termination of cell incubation, the medium and first rinse were pooled for collection of secreted proteins. Di-isopropylfluorophosphonate (Sigma Chemical Co., St. Louis, MO, U.S.A.), EDTA and SDS were added at final concentrations of 2 mM, 10 mM and 0.1% respectively, to denature proteins and inactivate proteinases. Non-incorporated radiolabelled amino acids were removed by exhaustive dialysis in dialysis tubing with a 3500 molecular mass cut-off (Spectrapor) against 50 mM-ammonium acetate. Samples were lyophilized and solubilized in an appropriate volume of SDS/PAGE sample buffer [12]. Secreted proteins derived from $8 \times 10^4$ cells of each sample were analysed by SDS/PAGE on a 7.5–15% gradient [12]. Human β2-microglobulin (11.8 kDa) was obtained from Sigma Chemical Co. Following electrophoresis, gels were dried and exposed to X-Omat AR film (Eastman-Kodak) for autoradiography. Densitometric measurement of protein bands was performed on a laser densitometer (LKB-XL, Pharmacia).

### Assay of collagenase activity

Collagenase activity was assayed by measuring degradation of [$^3$H]acetylated collagen fibrils [6] with media

Abbreviations used: IL-1, interleukin 1; TNF, tumour necrosis factor; DMEM, Dulbecco's modified Eagle medium; MEM, minimum essential medium; PAGE, polyacrylamide-gel electrophoresis.

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collected from cell monolayers, which were incubated in triplicate for 72 h as described above, in DMEM which contained no fetal calf serum. Assays were routinely performed in the presence of aminophenylmercuric acetate to activate collagenase. Data are shown as units secreted/$10^6$ cells where one unit of collagenase activity is defined as 1 $\mu$g of substrate degraded/min at 37 °C. Standard deviation between triplicate wells was equal to or less than 20% of the total c.p.m.

### *N*-Terminal sequence analysis

Conditioned media from IL-1 induced cells (lung fibroblasts and two synovial cell lines) were concentrated by approx. 50-fold by ultrafiltration using a YM5 membrane (Amicon), separated on SDS/PAGE, blotted to polyvinylidene difluoride membranes [13] and stained with Coomassie Brilliant Blue. The bands of interest (approx. 10 $\mu$g each) were excised and subjected to sequential Edman degradation on a gas phase sequenator (Applied Biosystems, 470A) following which the resulting phenylthiohydantoin amino acid derivatives were identified by an on-line Applied Biosystems 120A analyser.

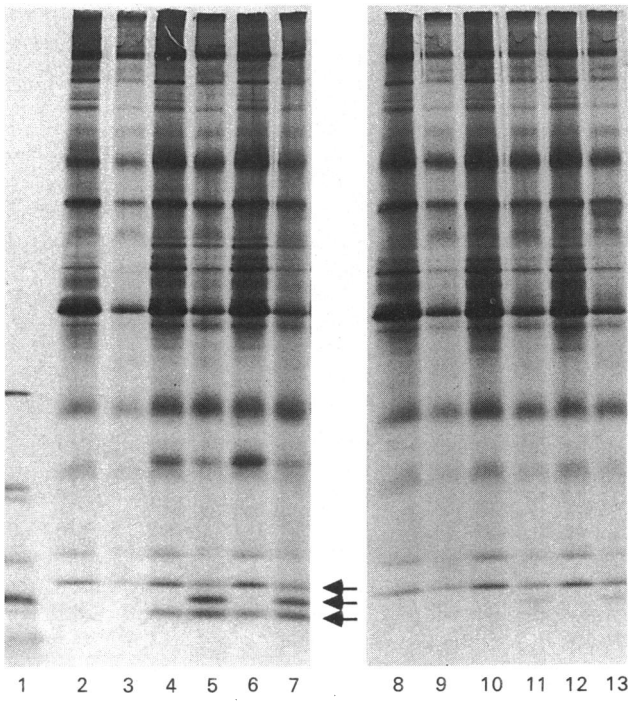


**Fig. 1. SDS/PAGE of $^{35}$S-labelled amino acid incorporation into proteins secreted by synovial cells**

Synovial cells incubated for 24 h in the presence of no additions (lanes 2 and 3), 0.2 U of IL-1$\beta$/ml (lanes 4 and 5), 2 U of IL-1$\beta$/ml (lanes 6 and 7), 0.4 ng of TNF/ml (lanes 8 and 9), 4.0 ng of TNF/ml (lanes 10 and 11) or 40 ng of TNF/ml (lanes 12 and 13). Cells were incubated with [$^{35}$S]methionine (lanes 2, 4, 6, 8, 10 and 12) or [$^{35}$S]cysteine (lanes 3, 5, 7, 9, 11 and 13). Collagenase activities detected were 0.05 U (non-induced cells), 2.3 U (0.2 and 2 U of IL-1), 0.8 U (0.4 ng of TNF), 0.9 U (4 ng of TNF) and 1.0 U (40 ng of TNF). Molecular mass standards (lane 1) from top to bottom are carbonic anhydrase (30 kDa), soybean trypsin inhibitor (a doublet, 21.5 kDa), cytochrome *c* (12.5 kDa) aprotinin (6.5 kDa) and insulin (3.5 kDa). The arrows on the right of lane 7 indicate, from top to bottom, the $M_r$-8000, -7000 and -6000 proteins.

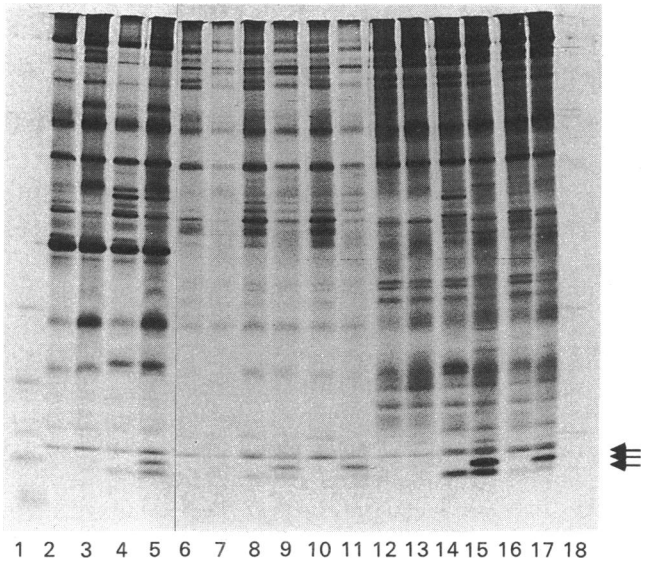


**Fig. 2. SDS/PAGE of $^{35}$S-labelled amino acid incorporation into proteins secreted by synovial cells, skin fibroblasts and lung fibroblasts**

Cells were incubated for 22 h in the presence of no additions (lanes 2, 3, 6, 7, 12 and 13), 5 U of IL-1/ml (lanes 4, 5, 8, 9, 14 and 15) or 20 ng of TNF/ml (lanes 10, 11, 16 and 17). Cells were incubated with [$^{35}$S]methionine (lanes 2, 4, 6, 8, 10, 12, 14 and 16) or [$^{35}$S]cysteine (lanes 3, 5, 7, 9, 11, 13, 15 and 17). Molecular mass standards in lane 1 from top to bottom are as for Fig. 1 and in lane 18 as follows: phosphorylase a (a doublet 92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). Proteins secreted by synovial cells (lanes 2–5), skin fibroblasts (lanes 6–11) and lung fibroblasts (lanes 12–17) were separated. The arrows on the right of lane 18 indicate, from top to bottom, the $M_r$-8000, -7000 and -6000 proteins.

The position of the cysteine residues was determined by using conditioned medium from cells induced in MEM containing [$^{35}$S]cysteine.

## RESULTS AND DISCUSSION

After IL-1 stimulation of synovial cells, cysteine-containing proteins with $M_r$ values of 6000 and 7000 were characteristically present on SDS/PAGE (Fig. 1). These two proteins migrated slightly ahead of a constitutively-secreted $M_r$-8000 protein. The $M_r$-7000 protein was not detected when cells were induced with IL-1 in the presence of [$^{35}$S]methionine. The $M_r$-6000 protein appeared to contain less [$^{35}$S]methionine than [$^{35}$S]cysteine (Fig. 1). Synthesis of these proteins was detected within 2 h of addition of IL-1 and sustained for at least 24 h (not shown). Prominent cysteine-rich $M_r$-6000 and -7000 proteins were not detected when cell-associated fractions of non-induced or induced cells were analysed on SDS/PAGE. The $M_r$-6000 and -7000 proteins were prominent IL-1-induction products in all synovial lines examined ($n = 7$) regardless of whether the cell line was derived from rheumatoid arthritic or osteoarthritic tissue. IL-1-induced expression of these two proteins was readily detected by Coomassie Brilliant Blue staining of 25-fold-concentrated cell medium separated on SDS/PAGE (not shown).

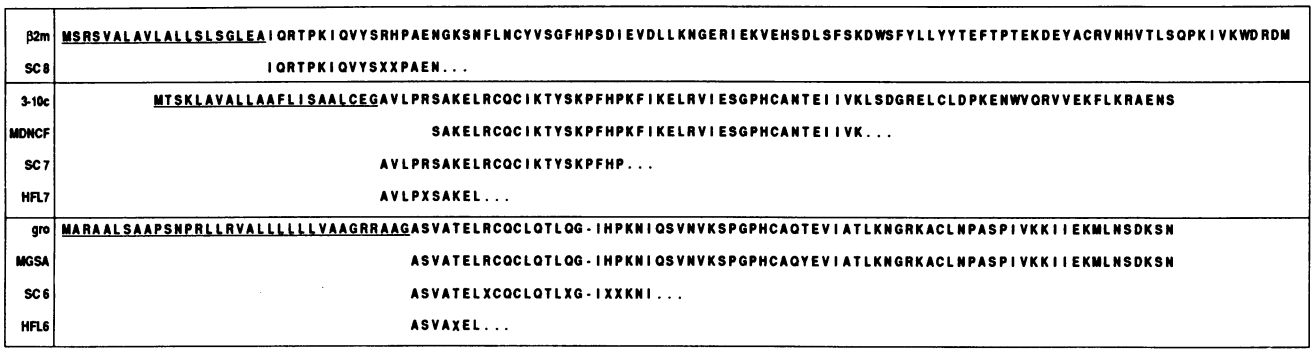


**Fig. 3. *N*-terminal amino acid sequences**

The sequences shown are for human β2-microglobulin (β2m) [14]; SC8, $M_r$-8000 from a synovial cell line; 3–10c as predicted from the cDNA [20]; MDNCF, the monocyte-derived neutrophil chemotactic factor [22]; SC7 and HFL7, $M_r$-7000 protein from synovial cells and lung fibroblasts, respectively; *gro* as predicted from the human cDNA [18]; MGSA, human melanoma cell protein [19]; SC6 and HFL6, $M_r$-6000 protein from synovial cells and lung fibroblasts. X, cycles in which no phenylthiohydantoin amino acid derivative could be identified.

Exposure of passaged human lung and skin fibroblasts to IL-1 also resulted in expression and secretion of $M_r$-6000 and -7000 proteins with characteristics similar to the synovial cell proteins (Fig. 2). In lung fibroblasts, IL-1-induction resulted in approximately equivalent expression of the $M_r$-6000 and -7000 proteins, whereas in skin fibroblasts, the $M_r$-7000 protein was synthesized and secreted to a greater extent than the $M_r$-6000 protein. An $M_r$-8000 protein was also constitutively secreted by lung and skin fibroblasts (Fig. 2).

The *N*-terminal sequence of the first 17 residues of the synovial cell $M_r$-8000 protein indicated that this protein was β2-microglobulin (Fig. 3) [14]. Commercially-obtained human β2-microglobulin comigrated with the $M_r$-8000 synovial cell protein on SDS/PAGE (results not shown). β2-Microglobulin appears to be a constitutively-secreted protein in synovial cells since densitometric analysis indicated that β2-microglobulin synthesis was increased by IL-1 and TNF only in proportion to cytokine-stimulation of overall protein synthesis (a range of 1.3–1.8-fold over controls for the cells shown in Fig. 1). β2-Microglobulin is a ubiquitous cell protein which is expressed on the cell surface as an integral component of the major histocompatibility type 1 complex [14]. However, recent reports indicate that it is also secreted from cells [15–17]. For example, β2-microglobulin is secreted from rat calvariae, where it has been proposed to modulate growth factor–receptor interactions [15,16]. Our data concur with those of Ramadori *et al.* [17] who have shown β2-microglobulin to be a secreted product of hepatocytes where its synthesis is induced by interferon but not IL-1 [17].

By *N*-terminal sequence analysis, the $M_r$-6000 protein was identified as the putative translation product of the *gro* mRNA (Fig. 3). This mRNA is expressed at high levels in a bladder carcinoma cell line and in virally-transformed, but not normal, human fibroblasts [18]. In human melanoma cells, the *gro* protein is an autocrine growth factor [19]. The *N*-terminal of the secreted $M_r$-6000 protein starts at Ala-35 which, based on the *gro* cDNA, indicates that the secreted protein should contain 73 amino acids (Fig. 3). The *N*-terminal of the secreted mitogenic melanoma protein also starts at Ala-35 (Fig. 3) [19]. IL-1-induction of the *gro* protein has not been previously reported.

Sequence analysis of the first 24 residues of the $M_r$-7000 protein indicated that it was the protein encoded by the 3–10c gene (Fig. 3) [20]. The 3–10c mRNA is expressed at high levels in human leukocytes stimulated with staphylococcal enterotoxin A [20]. According to the *N*-terminal sequence (Fig. 3) and the 3–10c cDNA, the $M_r$-7000 secreted protein starts immediately after the predicted signal peptide [20] and should contain 78 amino acid residues.

Human monocytes stimulated with lectins, lipopolysaccharide, IL-1 or TNFα have been recently shown to secrete the $M_r$-7000 protein which acts as a neutrophil chemotactic/activating factor [21–25]. This monocyte-derived protein has not been previously shown to be secreted from other cell types. The synovial cell and lung fibroblast $M_r$-7000 protein differs slightly from the monocyte-derived protein [21–23] by the presence of five additional *N*-terminal amino acids (AVLPR) which are identical to those predicted from the 3–10c cDNA starting immediately after the putative signal peptide [20]. The monocyte-derived protein, which was induced in media containing serum or plasma proteins [21–24], may be proteolytically cleaved after secretion to generate the slightly smaller protein. Although we can detect enhanced neutrophil chemotactic activity in synovial cell medium after IL-1-induction (P. Nyirkos & E. E. Golds, unpublished work) we do not know, at present, if this is due only to the $M_r$-7000 protein. While our work was under way, Watson *et al.* [26] reported that IL-1 stimulates release from human synovial cells of low molecular mass polymorphonuclear leukocyte-stimulating factors.

As shown in Fig. 3, the $M_r$-6000 and -7000 proteins each have four cysteine residues present in conserved positions. In agreement with [$^{35}$S]methionine incorporation data (Figs. 1 and 2), the $M_r$-7000 protein contains no methionine (Fig. 3). On the basis of structural similarity, the *gro* protein and the neutrophil chemotactic/activating protein are members of a newly-emerging superfamily of inflammation/transformation-related proteins [18–20,25]. This family includes a γ-interferon-induced protein, IP-10, which is expressed by human keratinocytes and other cell types [27], platelet factor 4 [28] and platelet basic protein [29], which is the precursor of connective tissue-activating peptide III [30] and β-thromboglobulin [29]. In chick-embryo fibroblasts,

viral transformation results in expression of an mRNA named either 9E3 [31] or pCEF4 [32]. The protein encoded by this mRNA likely represents the avian homologue of the $M_r$-7000 neutrophil-activating protein.

Since IL-1 and TNF share many properties, experiments were carried out to determine if the *gro* protein and neutrophil chemotactic protein were induced by TNF. The response of skin fibroblasts to TNF (Fig. 2, lanes 10 and 11) was similar to that observed with IL-1 (Fig. 2, lanes 8 and 9). In lung fibroblasts, TNF induction resulted in more prominent expression of the $M_r$-7000 protein, although the $M_r$-6000 protein was also induced (Fig. 2, lanes 16 and 17). Exposure of synovial cells to TNF at concentrations of up to 40 ng/ml resulted in barely detectable expression of these proteins (Fig. 1, lanes 8–13). Expression of collagenase activity in synovial cells was, however, stimulated by both cytokines (see legend to Fig. 1). The absence of a marked induction of the $M_r$-6000 and -7000 proteins by TNF in synovial cells was confirmed in four additional cell lines.

Co-expression of the *gro* protein and the neutrophil-activating protein has not been previously reported. In human synovial cells, IL-1 induction results in the striking expression of both proteins. However, expression of these proteins does not appear to be tightly co-regulated, since both IL-1 and TNF induction of skin fibroblasts results in more prominent expression of the $M_r$-7000 protein, and TNF preferentially induces expression of the $M_r$-7000 protein in lung fibroblasts. In response to TNF, synovial cells secrete very little of either protein, although collagenase activity is stimulated by both TNF and IL-1. Overall, both IL-1 and TNF induce the $M_r$-7000 and -6000 proteins. The degree to which these proteins are expressed and the relative amounts of each secreted vary depending on the cytokine-inducer and the type of mesenchymal target cell.

Because expression of the *gro* and 9E3/pCEF4 mRNAs are elevated during viral transformation [18,31,32], and because of the neutrophil-activating properties of the $M_r$-7000 monocyte-derived protein [21–25], it is possible that $M_r$-6000 and -7000 proteins act as secondary cytokines or local recruitment factors induced in mesenchymal cells by IL-1 and TNF during an inflammatory response. Their expression may be critical to events related to tissue turnover and remodelling such as occurs during wound healing and development. IL-1- and TNF-induced expression of the *gro* protein and the neutrophil-activating factor expands the list of properties attributed to these pleiotropic mediators of the immune response.

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