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Interleukin 1 and tumour necrosis factor increase phosphorylation of fibroblast proteins

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Interleukin 1 (IL1) or tumour necrosis factor (TNF) stimulated phosphorylation of a triad of 27 kDa phosphoproteins (p*I* 6.0, 5.7 and 5.5) in human dermal fibroblasts. The change was dependent on the dose of cytokine in the range 0.1–20 ng, was detectable between 3 and 5 min after stimulation and was maximal by 10 min. The proteins were found in the cytosol after subcellular fractionation. The ^{32}P was removed from them by alkali, indicating the presence of phosphoserine and/or phosphothreonine. The results suggest that early changes in serine/threonine protein kinase activity may be involved in responses of fibroblasts to IL1 and TNF.

Interleukin-1; Tumor necrosis factor; Protein phosphorylation; (Fibroblast)

1. INTRODUCTION

Interleukin 1 (IL1) and tumour necrosis factor (TNF) α are pro-inflammatory cytokines that have similar pleiotropic effects and act on many cell types. They are involved in local inflammatory responses, fever, the acute-phase response, immunoregulation and cachexia [1,2]. They act through high-affinity receptors but little is known of their post-receptor mechanisms. IL1 causes no change in phosphoinositide breakdown, intracellular Ca^{2+} or protein kinase C translocation in a T lymphoma line [3], although it does apparently increase breakdown of phosphatidylcholine (without affecting phosphatidylinositol) in Jurkat cells [4]. IL1 does not change cAMP levels in fibroblasts and chondrocytes [5].

Connective tissues are thought to be important targets of IL1 and TNF because they induce cartilage [6] and bone [7,8] resorption. They activate connective tissue cells directly to increase glycolysis [9] and the synthesis of a variety of products including prostaglandins, proteinases [1,2] and interleukin 6 [10]. Since protein phosphorylation is the major known mechanism by which extracellular signals regulate intracellular processes, we decided to investigate whether IL1 or TNF caused early changes in such phosphorylation in fibroblasts.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

Human dermal fibroblasts (passages 4–8) derived from infant foreskin were cultured in Dulbecco's modified medium containing 10% foetal calf serum (FCS) and maintained at 37°C in a humidified atmosphere of air enriched with 5% CO_2.

2.2. *Reagents*

Human recombinant IL1α (2×10^7 LAF units/mg) was from Dainippon (Osaka). Human recombinant TNFα (5×10^7 U/mg) was from Dr G.R. Adolf (Ernst Boehringer Institut, Vienna). [^{32}P]Orthophosphoric acid (8500–9120 Ci/mmol) was from Dupont/NEN.

The buffer for radiolabelling cells was phosphate-free Krebs-Ringer buffer containing 1% bovine serum albumin, 0.2% glucose and 20 mM Hepes. Ampholines were from LKB.

2.3. *Radiolabelling and stimulation of cells*

Newly confluent fibroblasts were washed in buffer (1.0 ml/30 mm diameter dish) for 20 min, then incubated with

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[^{32}P]orthophosphoric acid (100 μCi/ml) in fresh buffer (0.5 ml/dish) for 2 h at 37°C and then stimulated by adding IL1 or TNF. After further incubation for the required time, the labelling medium was rapidly removed, the cells quickly washed twice in ice-cold phosphate-buffered saline, and 0.5 ml of 3% trichloroacetic acid was added to each dish. The contents were scraped off and transferred to microcentrifuge tubes and centrifuged at 13000 rpm for 2 min: pellets were dissolved in 50 μl of 8 M urea/1% Nonidet P40 for focusing.

2.4. *Two-dimensional electrophoresis*

This was carried out according to O'Farrell [11]. Focusing gels contained 8 M urea, 3.5% total acrylamide, 4% ampholine (pH 5–7), 1% ampholine (pH 3–10), and 2% Nonidet P40. SDS-PAGE was carried out in a 12.5% separating gel.

Stained gels were dried and autoradiographed for 24–48 h with RX film (Fuji) with an image-intensifying screen. Marker proteins were: muscle phosphorylase (94 kDa), transferrin (78 kDa), albumin (68 kDa), IgG (50 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (21 kDa).

2.5. *Subcellular fractionation*

Confluent fibroblasts (2 × 175-cm^2 flasks) were stimulated with 5 ng/ml IL1 for 10 min, washed quickly in saline, then H_2O, incubated for 20 min in 10 ml swelling buffer (10 mM NaF, 10 mM NaH_2PO_4, 1 mM EDTA; pH 7.4) and finally broken in a small Dounce homogenizer (30 strokes). The NaF concentration was adjusted to 100 mM and the broken cells were centrifuged for 5 min at 400 × *g* to remove nuclei and intact cells. The supernatant was centrifuged for 1 h at 100000 × *g*. Proteins were precipitated from the final supernatant by adjusting it to 3% trichloroacetic acid. The fractions were analysed by two-dimensional electrophoresis.

3. RESULTS

When the cells were stimulated by a high dose of IL1 for 10 min there was a marked increase in phosphorylation affecting a triad of 27 kDa pro-

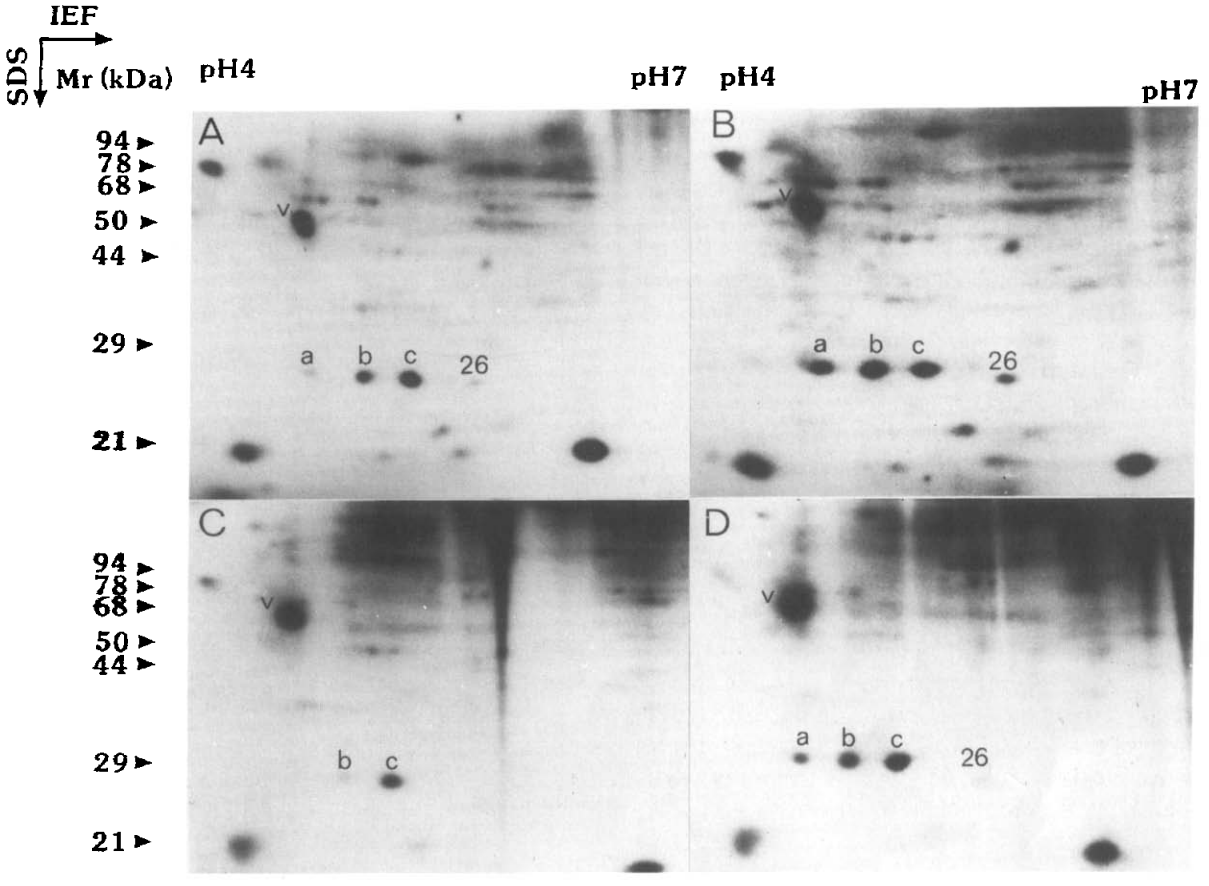

Fig.1. Autoradiographs of 2-D electrophoresis of phosphoproteins of human dermal fibroblasts. Expt 1: (A) control; (B) 20 ng/ml IL1α for 10 min. Expt 2: (C) control; (D) 100 ng/ml TNFα for 10 min. v, vimentin; a–c, the 27 kDa phosphoproteins; 26, the 26 kDa phosphoprotein.

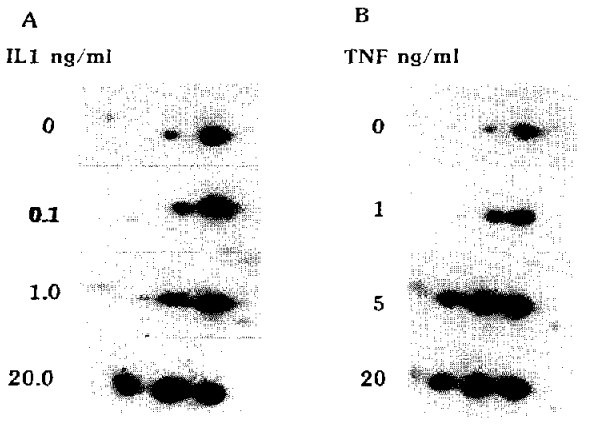

Fig.2. Change of phosphorylation of 27 kDa proteins with increasing dose of cytokines on human dermal fibroblasts for 10 min. The appropriate regions of autoradiographs of 2-D gels are shown. (A) IL1α; (B) TNFα.

teins (fig.1A,B). Two members, 27a and b (p*I* 5.5 and 5.7), showed a marked increase, while the third, 27c (p*I* 6.0), showed little change. Most of the major phosphoprotein spots had similar intensity when stimulated and unstimulated cells were compared, but there was some generalized increase in phosphorylation affecting a number of proteins in the range 40–70 kDa, and one of 26 kDa (p*I* 6.2). IL1α and β had similar effects. The change in the 27 kDa proteins was also induced by stimulating cells with a high dose of TNFα for 10 min (fig.1C,D). In this experiment, all three members of the 27 kDa triad showed increased phosphorylation, as did the 26 kDa protein. The generalized increases noted before were not seen. The specific activity of the radiolabelled pool of ATP in fibroblasts was not altered during stimulation with the cytokines (not shown). In a number of experiments carried out with either cytokine the increase in phosphorylation of the 27 kDa proteins was consistently observed, but the other changes were variable. We therefore decided to study this change.

The magnitude of the effect on the 27 kDa proteins was dependent on the dose of cytokine (fig.2). The increase in phosphorylation was detectable at 1 ng/ml of either cytokine, and reached a maximum by 20 ng/ml.

Investigation of the time dependence of the change showed that it was not detectable at 1 or 3 min after stimulation by 100 ng/ml TNF (fig.3A), but by 5 min there was a clear response which was maximal by 10 min. Longer stimulation periods (up to 30 min) showed no further change in intensity of the phosphorylation spots. A similar time dependence was found for IL1 (fig.3B), the change being detectable after 5 min, but not 3 min of stimulation.

Upon subcellular fractionation the 27 kDa

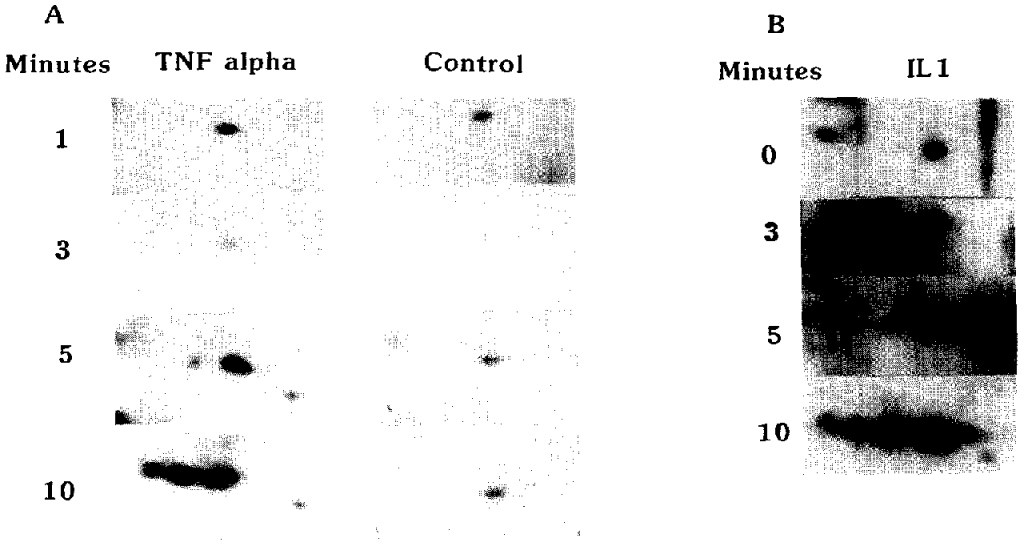

Fig.3. Autoradiographs showing the change of phosphorylation of the 27 kDa proteins with time in stimulated human dermal fibroblasts. (A) 100 ng/ml TNFα; a control in which no cytokine was added is shown for each time point; (B) 20 ng/ml IL1α.

phosphoproteins were located mainly in the 100000 × g supernatant (fig.4). It was calculated from densitometric scanning that 91% of the 27 kDa proteins recovered were in this fraction and 9% in the 100000 × g particulate fraction. The latter contained the 26 kDa phosphoprotein whose intensity increased with IL1 stimulation (fig.1A). The fractionation procedure did not preserve the integrity of lysosomes or mitochondria but the results were consistent with a cytosolic location of the 27 kDa triad.

Fixed and stained electrophoresis gels of stimulated labelled cells were soaked in 1 N NaOH for 2 h at 55°C before drying and autoradiography. This treatment removed the 27 kDa and many other phosphoprotein spots, indicating that they contained phosphoserine and phosphothreonine rather than phosphotyrosine, which is stable to alkali [12].

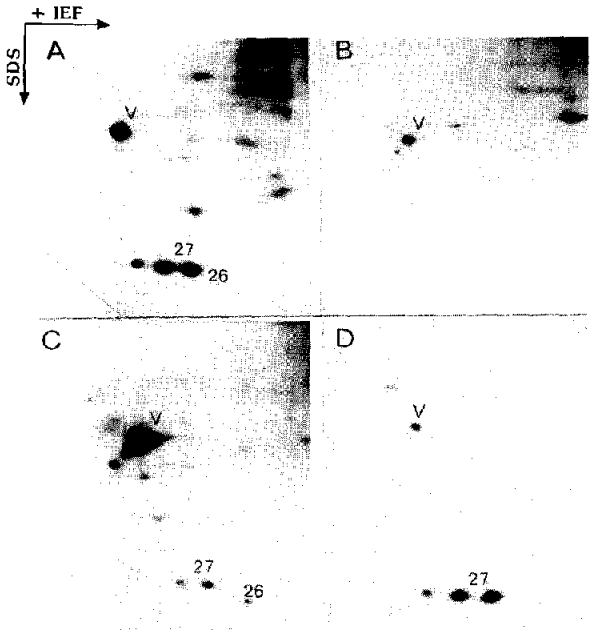

Fig.4. Autoradiographs of 2-D electrophoresis of subcellular fractions of human dermal fibroblasts. The cells had been labelled with [^{32}P]orthophosphoric acid and stimulated with IL1. (A) Whole cell extract; (B) 400 × g pellet (nuclei); (C) 100000 × g particulate fraction; (D) 100000 × g supernatant. Gel (D) was only loaded with half the relative amount of cellular material compared to gels (B) and (C). v, vimentin; 27 and 26 denote the 27 kDa and 26 kDa phosphoproteins, respectively.

4. DISCUSSION

IL1 and TNF induced a similar increase in phosphorylation of a triad of 27 kDa proteins. The concentration range over which this occurred (1–20 ng/ml) was within that needed to induce biological responses such as prostaglandin synthesis and cartilage resorption [6,7,13]. The effect was not rapid and apparently occurred between 3 and 5 min after stimulation: even at very high concentrations of cytokine (1 μg/ml IL1) the change was not seen earlier. This increase in phosphorylation is unlikely to be one of the earliest events in the signal cascade, but does indicate that changes in serine/threonine kinase activity are involved in the responses to the cytokines. The location of the kinases involved is not known but the substrate is probably cytosolic.

Whether the three 27 kDa phosphoproteins are different phosphorylation states of one molecule, or different proteins is not known. Their positions in the gels are similar to those of 3 phosphoproteins from rat embryo fibroblasts which were thought, on the basis of phosphopeptide mapping and immunoreactivity, to be phosphorylated isoforms of the cytosolic heat-shock protein hsp27 [14,15]. Phosphorylation of these was increased by treating cells with phorbol myristate acetate or calcium ionophore or serum for 1 h. We are investigating the relationship of the phosphoproteins reported here to hsp27. Increased phosphorylation of 27 kDa proteins has also been reported in Chinese hamster fibroblasts stimulated by serum or growth-factors [16], and bovine vascular endothelial cells treated with high density lipoprotein or phorbol ester [17].

IL1 has been reported to cause changes in phosphorylation of proteins in some other cell types; a 65 kDa cytosolic protein in glucocorticoid-treated mononuclear leucocytes [18], a 41 kDa membrane protein in K562 cells [19] and 52, 46 and 20 kDa membrane proteins in mesangial cells [20]. The K562 protein and the 46 kDa mesangial cell protein were phosphorylated on tyrosine, the others on serine and threonine.

Our observations are consistent with recent work from this laboratory (Bird, T.A. and Saklatvala, J., submitted) showing that IL1 or TNF transmodulate the epidermal growth factor receptor on fibroblasts. The kinetics of transmodulation

are similar to those reported here, and it is associated with increased serine and threonine phosphorylation of the receptor. Interestingly, the transmodulation apparently proceeds through a kinase C-independent mechanism.

Taken together our results provide direct evidence that IL1 and TNF cause early changes in serine/threonine kinase activity in stimulated fibroblasts.

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