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Mass spectrometric characterisation of an interleukin-1α-like polypeptide induced in keratinocytes by skin irritants

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Abstract

Stimulation of human epidermal keratinocytes with irritant phenolic compounds leads to the release of potent immunologically active mediators. A principal protein component released into the cell supernatant has been shown to have the biological and immunological properties appropriate for the primary cytokine interleukin-1-alpha. Electrospray mass spectrometry (ESI-MS) has been used to show that the mass of this protein corresponds to only a part of the published cDNA based sequence. ESI-MS has also been used to describe the secondary, immunologically active components, formed when fibroblasts are incubated with an extract of this protein. (Int J Mass Spectrom 188 (1999) 7–15) © 1999 Elsevier Science B.V.

Keywords: Skin; Interleukin; Chemokines; Electrospray mass spectrometry; Inflammation

1. Introduction

We have investigated the release of pro-inflammatory protein mediators from stimulated human keratinocytes and the effect of these on another immunologically active cellular component, dermal fibroblasts [1]. Immunological and inflammatory responses in human skin can be initiated by the release of interleukin-1 (IL-1) from keratinocytes [2,3]. IL-1 acts in both an autocrine and a paracrine manner to stimulate the release of biologically active lipid mediators such as prostaglandin E_2 [4] and a number of proteins including members of the large family of chemokines that determine, in a highly specific manner, which cells are recruited to the site of injury [3,5]. Identification and determination of IL-1 release have employed measurements of ribonucleic acid (mRNA), sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblotting experiments, enzyme linked immunosorbent assay (ELISA) and a variety of secondary biological assays [6–8].

IL-1 has been described in two molecular forms IL-1α and IL-1β with 26% sequence homology, both of which are synthesised as precursors of 31 and 34 kDa, respectively [9]. Whereas IL-1β is processed by well-characterised enzyme systems to a 17 kDa mature active protein [10,11], no similar proteolytic systems have been described for IL-1α. SDS-PAGE/immunoblotting experiments have suggested that two immunoactive forms of the molecule exist of approximately 31 and 17 kDa [12]. The literature also contains several reports of smaller biologically active IL-1α like molecules, found in a variety of sources, with sizes varying from 2 kDa upwards, which may

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represent proteolytic fragments of the parent species [13].

The skin contains substantial quantities of biologically active preformed IL-1α, the principal source being the keratinocyte [14]. Keratinocyte activation initiates an immune response in human skin by the immediate release of IL-1α. This study is designed to define the molecular nature of the IL-1α-like proteins released directly from keratinocytes by a phenolic irritant and also to confirm the biological activity of these by analysing the prostaglandin E_2 and chemokine molecules released on incubation of fibroblasts with these keratinocyte derived proteins. Electrospray mass spectrometry (ESI-MS) has played a key role in this exercise.

2. Experimental

2.1. Reagents

Dulbecco's modified Eagles medium (DMEM), heat inactivated foetal bovine serum, 2 mM L-glutamine, 100 U/L penicillin, 0.2 mg/L streptomycin supplement, and 0.25% trypsin solution were purchased from Sigma, Poole, UK. Keratinocyte serum free medium (KSFM) containing 5 μL/mL bovine pituitary extract and 5 ng/mL epidermal growth factor was purchased from Life Technologies, Paisley, UK. Recombinant human interleukin-1α (rhIL-1α) was obtained from NIBS, Herts, UK. Acetonitrile and glacial acetic acid (Analytical grade) were purchased from Fisher Scientific, Loughborough, UK. Trifluoroacetic acid (TFA), 0.880 ammonia solution, and Tris base were obtained from Sigma, Poole, UK.

2.2. Cell culture preparation

2.2.1. Keratinocytes

Human epidermal keratinocytes were isolated and maintained from skin circumcisions as described elsewhere [1,15]. Cultures were grown in KSFM with 5 μL/mL bovine pituitary extract, 5 ng/mL epidermal growth factor plus 2 mM L-glutamine, 100 U/L penicillin, 0.2 mg/L streptomycin, and passaged by using trypsin. Cells after three passages were grown in T75 culture flasks with 10 mL media until 95% confluence. Keratinocytes were stimulated with 1.2 mM chloroxylenol in fresh medium and supernatants were removed after 8 h for analysis.

2.2.2. Fibroblasts

Human dermal fibroblasts were isolated from skin circumcisions and maintained as described by Sly and Grubb [16]. Cultures were grown in DMEM supplemented with 10% heat inactivated foetal bovine serum plus 2 mM L-glutamine, 100 U/L penicillin, 0.2 mg/L streptomycin and passaged by using trypsin. For chemokine identification, cells between passage four and six were grown in T75 culture flasks with 10 mL media until 90% confluence. Cells were stimulated by replacing the growth media with 9 mL of serum-free DMEM plus 1 mL of either KSFM (control) or KSFM containing 1000 pg/mL IL-1α. Cell culture supernatants were removed at 8 h. For kinetic studies, fibroblasts from the third passage were seeded in 12 well culture plates with 1 mL culture medium and grown to 90% confluence. Cells were stimulated by replacing the growth media with 0.9 mL of serum-free DMEM plus 100 μL KSFM containing 1000 pg/mL IL-1α. IL-1α treatment was omitted for the control. Cell culture supernatants were removed at 1, 4, 8, and 24 h for chemokine ELISA assays. Extraction of whole keratinocytes was performed by the method described by Blanton et al. [7]. In brief, the keratinocyte medium was replaced by 0.1% Triton X-100 in phosphate buffered saline (PBS). After centrifugation, acidification, and filtration through a 0.2 μ Millipore syringe filter (Waters, Watford, UK), the supernatant was concentrated 25-fold by passing through a 10 kDa membrane centrifuge concentrator (Amicon, Beverly, MA).

2.3. Isolation of keratinocyte derived IL-1α polypeptides

The supernatants were collected, acidified with glacial acetic acid (10 μL per 10 mL medium), cooled, and filtered through a 0.2 μm Millipore syringe filter (Waters, Watford, UK). The filtrate was

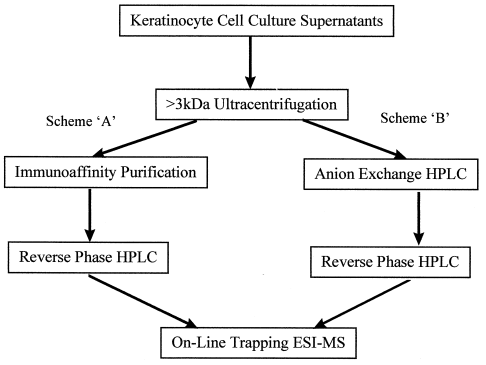

Fig. 1. IL-1α purification procedures. Alternative methods of keratinocyte supernatant IL-1α purification. Scheme A is by immunoaffinity chromatography, scheme B by dual mechanism HPLC.

concentrated by ultrafiltration through a 3 kDa membrane centrifuge concentrator (Amicon, Beverly, MA) and subjected to either immunoaffinity or HPLC purification.

2.3.1. Immunoaffinity purification

Rabbit antihuman IL-1α (Sigma) was immobilized on an agarose gel by using a CarboLink kit (Pierce and Warriner, UK). The gel was equilibrated with elution and washing solutions prior to use. The >3 kD retentate of keratinocyte supernatants was neutralised with 1 M Tris pH 9.5, applied to the affinity column and the antigen left to bind for 1 h. The column was washed with 14 mL of PBS to remove unbound proteins and then eluted with 0.1 mM glycine pH 2.5. 1 mL fractions were collected and those containing immunoactive (by ELISA) IL-1α were collected for purification as below.

2.3.2. HPLC purification

The >3 kDa ultrafiltration retentate of stimulated keratinocytes was neutralised with 1 M Tris pH 9.5 buffer and chromatographed on a 10 cm × 1 mm i.d Perkin Elmer Brownlee AX300 anion exchange HPLC column. Eluent A was 20 mM ammonium acetate pH 8.0, eluent B 20 mM ammonium acetate pH 4.0. A linear gradient at 1.0 mL/min from 100%A to 98%A over 5 min was followed by a linear gradient to 100%B at 15 min and maintained at 100%B until

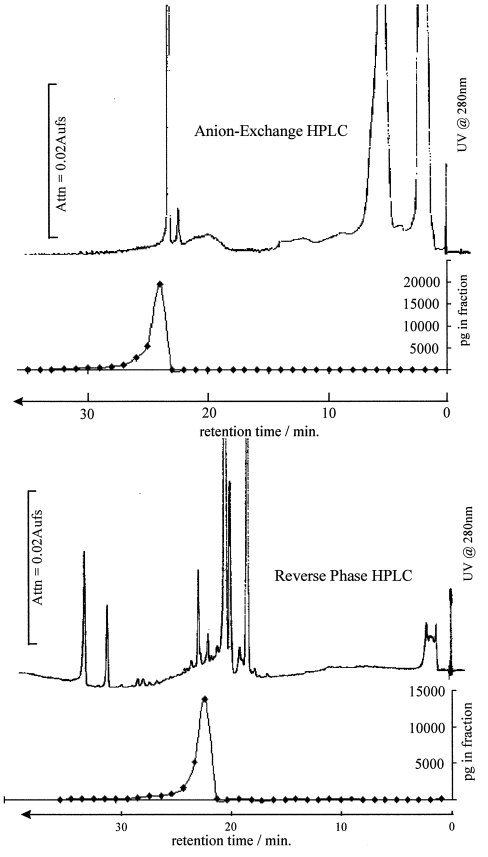

Fig. 2. HPLC purified of IL-1α. Anion exchange (top) and reverse phase (bottom) HPLC UV @ 280 nm and ELISA profiles for the >3 kDa fraction derived from keratinocytes after stimulation for 8 h by chloroxylenol.

40 min. Forty 1 mL fractions were collected and assayed for IL-1α activity by ELISA.

Reverse-phase HPLC was performed on both the anion-exchange and affinity purified IL-1α immunoactive fractions. Samples were applied to a 10 cm × 1 mm i.d Perkin Elmer Brownlee C_8 RP300 column and eluted at 1 mL/min. Eluent A was 10% acetonitrile/H_2O + 0.1% trifluoroacetic acid (TFA), eluent B 90% acetonitrile/H_2O + 0.1% TFA. Eluent A was maintained for 5 min followed by a linear gradient to 100%B at 35 min. Thirty-five 1 mL fractions were collected and assayed for the IL-1α-like material by ELISA.

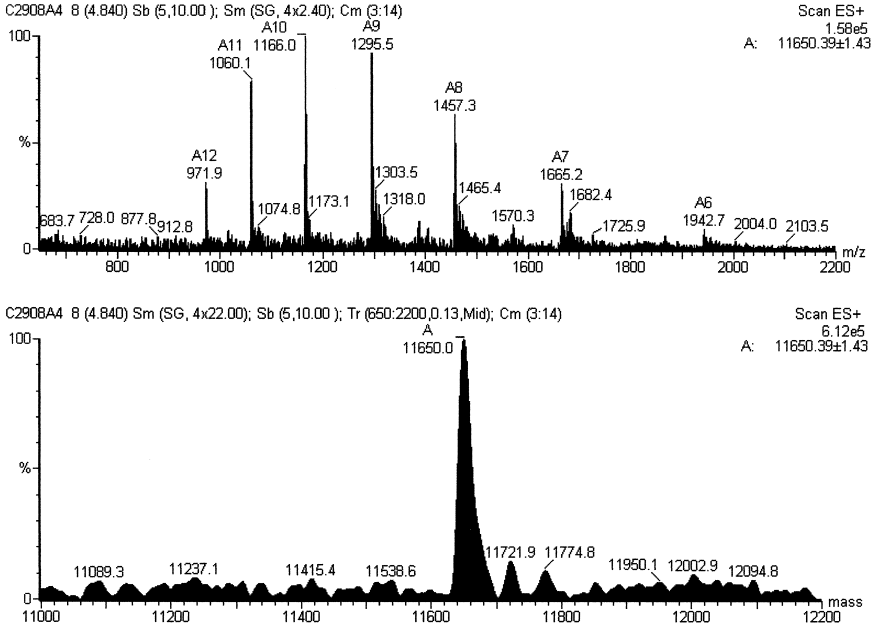

Fig. 3. Immunoaffinity purified IL-1α. ESI+ (top) and transformed (bottom) MS of immunoactive, affinity purified IL-1α fraction derived from keratinocytes after stimulation with a toxic dose of chloroxylenol for 8 h. A protein of mass 11 650 $\pm$ 1.4 Da was observed.

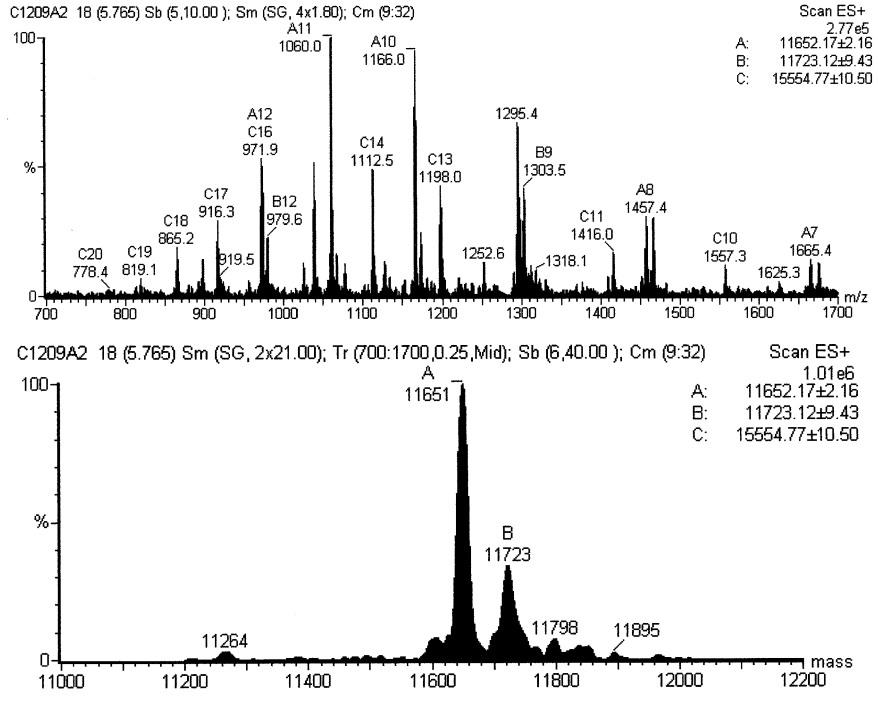

Fig. 4. HPLC purified IL-1α. ESI+ (top) and transformed (bottom) MS of HPLC purified IL-1α fraction derived from keratinocytes after stimulation with a toxic dose of chloroxylenol for 8 h. A protein mass 11 652 $\pm$ 1.2 Da was observed.

Table 1
Masses determined by ESI-MS of IL-1α fragment isolated

Cell source	Stimulus	Purification route (Fig. 1)	Mass (Da)
Keratinocyte line 1	Chloroxylenol 1.2 mM	A	11 650.4 ± 1.4
Keratinocyte line 2	Chloroxylenol 1.2 mM	B	11 652.2 ± 2.2
Keratinocyte line 3	Chloroxylenol 1.2 mM	A	11 652.6 ± 1.6
Keratinocyte line 1	SDS 440 μM	B	11 650.4 ± 0.9
		Mean	11 651.4 ± 0.8

2.4. Purification of fibroblast derived chemokine proteins

The procedures followed were those previously described by Kay et al. [17]. Fibroblast cell culture supernatants were collected and ultrafiltered through 50 kDa membrane concentrators (Amicon, Beverly, MA). The filtrates were then concentrated by using 3 kDa ultracentrifugation filters. The retentates (<2 mL) were applied to an immobilized heparin column (Pierce and Warriner, Chester, UK), left to bind for 30 min, washed with 14 mL 50 mM Tris pH 8.0 and the bound proteins eluted with 5 mL 0.65 M sodium chloride solution. This solution was desalted and concentrated to 1 mL by using 3 kDa ultracentrifugation concentrators for RP-HPLC purification. Samples were applied to a 10 cm × 1 mm i.d Perkin Elmer Brownlee C_8 RP300 column. Eluent A was 10% acetonitrile/H_2O + 0.1% TFA, eluent B was 70% acetonitrile/H_2O + 0.1% TFA. Eluent A was maintained isocratically for 5 min followed by a linear gradient to 100% B at 35 min with the flow rate maintained at 1.0 mL/min. Fractions corresponding to the appearance of UV absorbing peaks at $\lambda = 214$ nm were collected.

2.5. Electrospray ionization mass spectrometric characterisation

ESI-MS protein spectra were obtained by using an on-line trapping system as described by Kay and Mallet [18]. Briefly, a microbore protein trapping cartridge (Jones Chromatography, Clywd, UK) was used to capture the purified protein extract whilst any salts or TFA, which may have caused ESI-MS signal reduction [18], were removed with 10 parts acetonitrile + 90 parts water + 0.1 parts formic acid. The trapped proteins were then introduced into the ion

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1   Met-Ala-Lys-Val-Pro-Asp-Met-Phe-Glu-Asp-Leu-Lys-Asn-Cys-Tyr-Ser-Glu-Asn-Glu-Glu-
21  Asp-Ser-Ser-Ser-Ile-Asp-His-Leu-Ser-Leu-Asn-Gln-Lys-Ser-Phe-Tyr-His-Val-Ser-Tyr-
41  Gly-Pro-Leu-His-Glu-Gly-Cys-Met-Asp-Gln-Ser-Val-Ser-Leu-Ser-Ile-Ser-Glu-Thr-Ser-
61  Lys-Thr-Ser-Lys-Leu-Thr-Phe-Lys-Glu-Ser-Met-Val-Val-Val-Ala-Thr-Asn-Gly-Lys-Val-
81  Leu-Lys-Lys-Arg-Arg-Leu-Ser-Leu-Ser-Gln-Ser-Ile-Thr-Asp-Asp-Asp-Leu-Glu-Ala-Ile-
101 Ala-Asn-Asp-Ser-Glu-Glu-Glu-Ile-Ile-Lys-Pro-Arg-*Ser-Ala-Pro-Phe-Ser-Phe-Leu-Ser-
121 Asn-Val-Lys-Tyr-Asn-Phe-Met-Arg-Ile-Ile-Lys-Tyr-Glu-Phe-Ile-Leu-Asn-Asp-Ala-Leu-
141 Asn-Gln-Ser-Ile-Ile-Arg-Ala-Asn-Asp-Gln-Tyr-Leu-Thr-Ala-Ala-Ala-Leu-His-Asn-Leu-
161 Asp-Glu-Ala-Val-Lys-Phe-Asp-Met-Gly-Ala-Tyr-Lys-Ser-Ser-Lys-Asp-Asp-Ala-Lys-Ile-
181 Thr-Val-Ile-Leu-Arg-Ile-Ser-Lys-Thr-Gln-Leu-Tyr-Val-Thr-Ala-Gln-Asp-Glu-Asp-Gln-
201 Pro-Val-Leu-Leu-Lys-Glu-Met-Pro-Glu-Ile-Pro-Lys-Thr-Ile-Thr-Gly-Ser-Glu-Thr-Asn-
221 Leu-Leu-Phe-Phe-Trp-Glu-Thr-His-Gly-Thr-Lys-Asn-Tyr-Phe-Thr-Ser-Val-Ala-His-Pro-
241 Asn-Leu-Phe-Ile-Ala-Thr-Lys-Gln-Asp-Tyr-Trp-Val-Cys-Leu-Ala-Gly-Gly-Pro-Pro-Ser-
261 Ile-Thr-Asp-Phe-Gln-Ile-Leu-Glu-Asn-Gln-Ala
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Fig. 5. "Best fit" amino acid sequence the IL-1α polypeptide. The amino acid sequence for the IL-1α derived from monocyte cDNA sequencing (March et al., 1986). The active "mature" portion is reported to be the 159 amino acid sequence from position 113 (*Ser) to the C terminus. The only sequence to match our 11 651 Da fragment in this active portion is between amino acids 154 to 257 (shaded background).

source by flushing the column with a running solvent of 65 parts acetonitrile + 35 parts water + 0.1 parts formic acid at 7.5 μL/min. ESI-MS were collected on a Platform I spectrometer (Micromass, Wythenshawe, UK), with a source temperature of 90 °C, capillary 3.6 kV, and by using a cone voltage linear gradient of 37 V @ $m/z = 700$ and 125 V @ $m/z = 2200$. This was found experimentally to give the widest possible range of ions over the m/z scale for the calibrant protein horse heart myoglobin (Sigma, Poole, UK). Data was collected and processed by using MassLynx v2.2.

2.6. PGE2 analyses

Prostaglandin E2 was analysed as described [19]. In brief, deuterated PGE2 was added to cell supernatant samples (1.0 mL), these were centrifuged, purified through a C-18 reversed phase solid phase extraction cartridge (Isolute, Jones Chromatography, UK), and chromatographed with a gradient of 0.02% formic acid containing from 28% acetonitrile to 90% acetonitrile on a 1 mm × 15 cm Spherisorb ODS-2 column (Phase Separations, UK). This column was directly interfaced to a Platform I MS running in negative ion electrospray mode.

2.7. IL-1α and Chemokine ELISA

IL-8, GROα and MCP-1 ELISAs were obtained from R&D systems (Oxon, UK). Supernatants were diluted in DMEM and used directly in the ELISA as per the manufacturer's instructions. Recombinant human IL-1α was used to create the quantitative calibration curve.

3. Results and discussion

3.1. Characterisation of an IL-1α-like polypeptide fragment

Interleukin-1 is considered to be the principal protein molecule responsible for mounting an immunological response to an injury in skin. This protein is found prestored in several cell types and its release takes place as an immediate response to the stimulus. Following interaction of IL-1 with its receptor, either on the same cell or on other dermal cells such as fibroblasts, a number of immunologically active molecules are synthesised and released leading to the typical characteristics of a so-called inflammatory response.

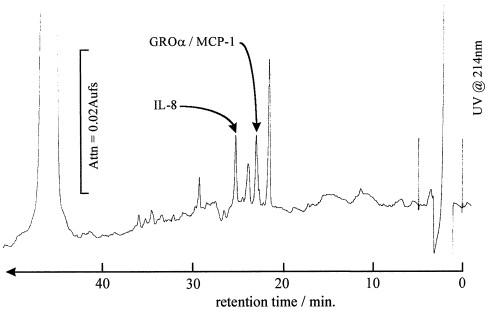

Fig. 6. HPLC purification of fibroblast chemokines. Reverse phase HPLC UV = 214 nm trace for chemokines derived from fibroblasts after stimulation with chloroxylenol treated keratinocyte conditioned media.

We have shown that keratinocytes treated for 8 h with a dose of chloroxylenol just sufficient to kill the cells (1.2 mM), leads to the release of immunoactive IL-1α-like material [1]. In order to determine the nature of the IL-1α-like molecule released we collected supernatants from chloroxylenol stimulated keratinocytes and purified them by one of two alternative schemes as shown in Fig. 1. Approximately 3 pmol of IL-1α-like protein was obtained from the supernatants collected from ten T75 flasks. The chromatographic separations from the two HPLC systems with ELISA results are shown in Fig. 2. Samples sufficiently pure for analysis by ESI-MS were obtained by both of these procedures when the immunoactive solutions were introduced to the mass spectrometer through an on-line trap system [18]. Figs. 3 and 4 show the spectra from two preparations and Table 1 summarises the masses we obtained from four distinct cell sources.

Fig. 5 shows the full cDNA derived IL-1α sequence. It is accepted that the first 112 residues are cleaved on release of the stored protein and that the active sequence is represented by the subsequent 159

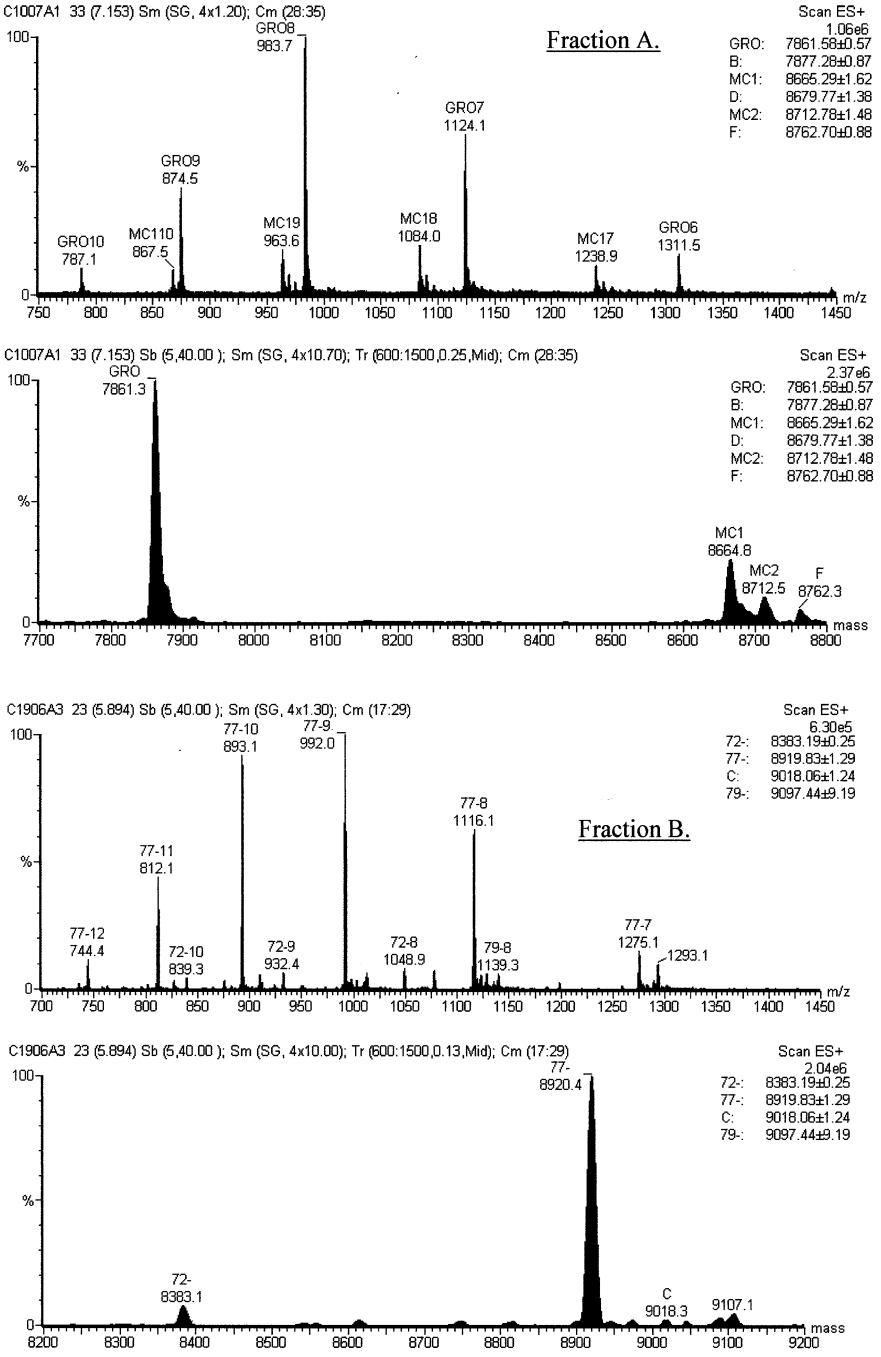

Fig. 7. Fibroblast chemokine identification. ESI+ (top) and transformed (bottom) mass spectra from heparin binding reversed phase HPLC purified fibroblast cell culture supernatant fractions. Fibroblasts were stimulated with media from keratinocytes that had been challenged with chloroxylenol for 8 h. Fraction A contained two principal proteins of mass 7861 and 8664 Da. These correspond to GROα and the pyroglutamic acid form of MCP-1 by weight. Fraction B contained the 72, 77, and 79 amino acid forms of IL-8. The fractions were also active for these proteins by ELISA.

residues [9]. Our protein's mass matches to ±1.0 Da the sequence from residues 154 to 257, the shaded region in Fig. 5. This does not correspond with the literature value of 17 kDa for the "active fragment" of IL-1α, but does represent a proteolytic cleavage of the full sequence at positions 154 to 257. In addition the estimated pI of this fragment, 5.2 agrees well with that derived from the elution volume on ion-exchange chromatography. In addition to this principal protein several other minor components were seen in the extracts. None of these was consistently present nor did they correspond in mass to a sequence in the mature IL-1α protein. Further work is in progress to sequence part of this fragment to confirm our conclusions. SDS-PAGE/immunoblotting of keratinocyte cell supernatants or immunopurified extracts did not show a band corresponding to this protein, however, a 31 kDa band was observed from extracts of whole keratinocytes, consistent with published observations.

3.2. Biological activity of the IL-1α-like polypeptide

Evidence for the appropriate biological activity of our IL-1α extracts has included its effect on human dermal fibroblasts. After a period of 8 h incubation the latter release a mixture of chemokine proteins. Treatment of one T75 flask ($\sim 1 \times 10^7$ cells) led to the release of approximately 50 pmol IL-8 equivalent. Chemokines are highly specific cellular chemoattractants determining which immunologically active cell types are recruited to the site of injury [5]. They are all small proteins of between 7 and 12 kDa and are, with a few exceptions, not post-translationally modified. Mass spectrometry has been shown to be an important tool in the molecular definition of these proteins [17] and we have used a procedure based on the common property of all chemokines to be heparin binding molecules in order to analyse the chemokine product profile of fibroblasts following stimulation by our keratinocyte derived IL-1α material. Fig. 6 shows a RPHPLC profile for a heparin immunoaffinity extract of the cell. Two fractions from this, on analysis by ESI-MS, showed the presence of three principal chemokine proteins, GRO-α, MCP-1 in its pyroglutamic acid form, and three forms of IL-8

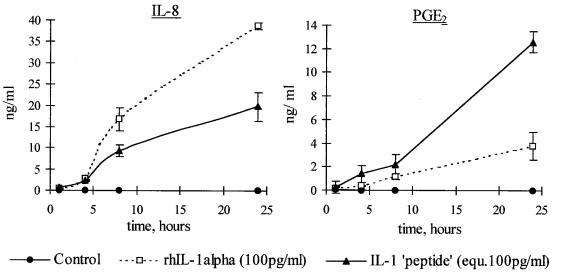

Fig. 8. Chemokine profile of fibroblasts stimulated by interleukin-1α materials. Kinetic profiles for IL-8 and PGE_2 after stimulation of fibroblasts by an immunoequivalent dose of recombinent human IL-1α and the immunopurified keratinocyte derived IL-1α.

differing only in the lengths of the N-terminal sequence (Fig. 7). The presence of these three chemokines was confirmed by their positive response to the appropriate commercial ELISA assays. The release of PGE_2 on IL-1α stimulation of fibroblasts is well documented [4] and we have determined the kinetic profiles of IL-8 and PGE_2 release [19] after stimulation of fibroblasts by both rhIL-1α and our immunopurified IL-1α-like polypeptide. Very similar curves were obtained for both the recombinant protein and for our extracts (Fig. 8).

It appears that, in keratinocytes at least, IL-1α is processed to a newly described biologically active fragment. In stimulated keratinocyte supernatants, we have been unable to detect any of the whole protein (31 kDa) or the 17 kDa fragment usually described, either by mass spectrometry or by SDS-PAGE experiments. In the case of the latter we may have had too little material for successful detection, although SDS-PAGE analysis of a whole keratinocyte extract showed a 31 kDa band agreeing with the literature. The close agreement of the masses obtained for the polypeptide fragment from a number of sources would appear to rule out artefactual proteolysis during sample workup and points to the existence of specific proteolytic pathways that may operate in keratinocytes that are stimulated with a toxic dose of an irritant chemical, leading to the release of a biologically active IL-1α fragment. The value of electrospray mass spectrometry when combined with modern bioassay, immunoaffinity, and chromatographic proce-

dures in determining the precise structure of biologically important proteins is clearly demonstrated in this investigation.

Acknowledgement

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References

[1] C.S. Newby, R.M. Barr, M.W. Greaves, A.I. Mallet, J. Invest. Dermatol. 109 (1997) 453 (abstract).
[2] T.S. Kupper, J. Clin. Invest 86 (1990) 1783.
[3] I.R. Williams, T.S. Kupper, Life Sciences 58 (1996) 1485.
[4] R.M. Barr, P.H. Symonds, A.S. Akpan, M.W. Greaves, Exp. Cell Res. 198 (1992) 321.
[5] T.A. Springer, Cell 76 (1994) 301.
[6] J.L. Wilmer, F.G. Burleson, F. Kayama, J. Kanno, M.I. Luster, J. Invest. Dermatol. 102 (1994) 915.
[7] R.A. Blanton, T.S. Kupper, J.K. McDougall, S.K. Dower, Proc. Natl. Acad. Sci. (USA) 86 (1989) 3045.
[8] R.D.R. Camp, N. Fincham, J. Ross, C. Bird, A. Gearing, J. Invest. Dermatol. 94 (1990) 735.
[9] C.J. March, B. Mosley, A. Larsen, D.P. Corretti, G. Braedt, V. Price, S. Gillis, C.S. Henney, S.R. Kronheim, K. Grabstein, P.J. Conlon, T.P. Hopp, D. Cosman, Nature 315 (1985) 641.
[10] T.S. Kupper, R.W. Groves, J. Invest. Dermatol. 105 (1995) 62s.
[11] H. Mizutani, R. Black, T.S. Kupper, J. Clin. Invest. 87 (1991) 1066.
[12] K.A. Hoquist, M.A. Nett, E.R. Unanue, D.D. Chaplin, Proc. Natl. Acad. Sci. (USA) 88 (1991) 8485.
[13] J.J. Oppenheim, E.J. Kovacs, K. Matsushima, S.K. Durum, Immunol. Today. 7 (1986) 45.
[14] A. Corradi, A.T. Franzi, A. Rubartelli, Exp. Cell Res. 217 (1995) 355.
[15] R. Mitra, B.J. Nickoloff in I.M. Leigh and F.M. Watt (Eds.), Keratinocyte Methods, Cambridge University Press, Cambridge, 1994, p. 17.
[16] W.S. Sly, J. Grubb in W.B. Jakoby and I.H. Pastan (Eds.), Methods in Enzymology, Academic, San Diego, 1979, Vol. 58, p. 444.
[17] I. Kay, S. Hafizi, R.M. Barr, A.I. Mallet, M.W. Greaves, Biol. Mass Spec. 23 (1994) 267.
[18] I. Kay, A.I. Mallet, Rapid Commun. Mass Spectrom. 7 (1993) 744.
[19] C.S. Newby, A.I. Mallet, Rapid Commun. Mass Spectrom. 11 (1997) 1723.