Expression of transcripts for two interleukin 8 receptors in human phagocytes, lymphocytes and melanoma cells

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Two cDNAs coding for distinct interleukin 8 (IL-8) receptors, IL-8R₁ [Murphy and Tiffany (1991) *Science* **253**, 1280–1283] and IL-8R₂ [Holmes, Lee, Kuang, Rice and Wood (1991) *Science* **253**, 1278–1280] have been reported, and biochemical studies on human neutrophils have revealed two proteins (p70 and p44) that bind IL-8 with high affinity [Moser, Schumacher, von Tschanner, Clark-Lewis and Baggiolini (1991) *J. Biol. Chem.* **266**, 10666–10671]. We have cloned the cDNA coding for IL-8R₁ from a library of differentiated HL-60 cells. Transfection of this cDNA into Jurkat cells resulted in the expression of high-affinity binding for IL-8 and two related cytokines, GRO α and neutrophil-activating peptide 2 (K_d 0.5–1.0 nM). Northern-blot analysis with the IL-8R₁ cDNA as probe revealed abundant expression of transcripts of different size in human neutrophils and low-level expression of a single RNA species in HL-60 cells differentiated with dimethyl sulphoxide and retinoic acid. Because of the extensive nucleotide sequence similarity of the cDNAs for IL-8R₁ and IL-8R₂, the reverse-transcription PCR method was

used for analysis of RNA expression in myeloid and lymphoid cells, 19 cell lines established from human primary melanomas or metastases, two melanocyte and one fibroblast cell lines. IL-8R₁ mRNA transcripts were expressed at high levels in neutrophils, and to a lesser extent in blood monocytes and the myeloid cell lines, HL-60 and AML 193, but were not found in THP-1 cells, lymphocytes and Jurkat cells. IL-8R₂ mRNA transcripts, by contrast, were found in all blood cells and related cell lines, as well as in all melanoma, melanocyte and fibroblast cell lines tested. As for IL-8R₁, IL-8R₂ mRNA expression was highest in neutrophils. These results suggest that IL-8R₁ and IL-8R₂ may both be involved in neutrophil activation by IL-8 and related cytokines, and presumably correspond to p70 and p44, the receptors that were identified biochemically. Possible IL-8 functions on lymphocytes and melanoma cells, e.g. chemotaxis and proliferation, must be independent of IL-8R₁ and may be mediated by IL-8R₂.

INTRODUCTION

Interleukin 8 (IL-8) and two structurally related cytokines, GRO α and neutrophil-activating peptide 2 (NAP-2), were shown to induce chemotaxis and activation of neutrophil leucocytes (Baggiolini et al., 1989; Oppenheim et al., 1991; Baggiolini and Clark-Lewis, 1992). A wide variety of tissue cells, in addition to blood leucocytes, produce IL-8 and GRO α after stimulation with inflammatory cytokines such as interleukin 1 and tumour necrosis factor (TNF), suggesting that these chemotactic cytokines are major mediators of inflammation (Baggiolini et al., 1989; Oppenheim et al., 1991; Baggiolini and Clark-Lewis, 1992). Neutrophil leucocytes are recognized as the primary target cells for IL-8 and related cytokines on the basis of numerous studies describing mobilization of intracellular Ca²⁺, shape change, chemotaxis, release of granule enzymes, upregulation of adhesion receptors and the respiratory burst (Peveri et al., 1988; Colditz et al., 1989; Walz et al., 1989, 1991b; Moser et al., 1990; Walz and Baggiolini, 1990; Detmers et al., 1991; Leonard et al., 1991; Swensson et al., 1991). Actions on other cells have also been found, however, IL-8 elicits Ca²⁺ changes and the respiratory burst in monocytes (Walz et al., 1991a), and induces the release of histamine and leukotrienes in basophil leucocytes (Dahinden et al., 1989). IL-8 was also reported to be chemotactic for lymphocytes (Larsen et al., 1989), to induce proliferation of

keratinocytes (Krueger et al., 1990) and to attract melanocytes *in vitro* (Wang et al., 1990). In addition, GRO α was described as a mitogen for melanoma cells, and was originally termed melanoma growth-stimulatory activity (MGSA) (Anisowicz et al., 1988; Richmond et al., 1988).

Studies from several laboratories have shown that neutrophils express high-affinity receptors for IL-8 (K_d 0.2–4 nM; up to 75000 sites per cell) (Besemer et al., 1989; Samanta et al., 1989, 1990; Grob et al., 1990; Moser et al., 1991). In cross-linking experiments, two proteins of 44 kDa (p44) and 70 kDa (p70) became specifically labelled with radioiodinated IL-8, GRO α and NAP-2 (Moser et al., 1991; Schumacher et al., 1992). IL-8 binds to both receptors with high affinity, whereas GRO α and NAP-2 interact with high affinity (K_d 0.3–0.7 nM) with p70 and low affinity (K_d 30 nM) with p44 (Schnitzel et al., 1991; Schumacher et al., 1992). These observations suggest that all three cytokines activate neutrophils via common receptors.

The cDNAs of two IL-8 receptors, termed here IL-8R₁ (Murphy and Tiffany, 1991) and IL-8R₂ (Holmes et al., 1991), were recently cloned and shown to code for seven-transmembrane-domain G-protein-coupled receptors. COS-7 cells transfected with IL-8R₂ cDNA expressed high-affinity binding sites for IL-8, but similar transfection studies were not performed with the cDNA coding for IL-8R₁ (Murphy and Tiffany, 1991; Holmes et al., 1991). Using Northern-blot analysis

Abbreviations used: IL-8, interleukin 8; NAP-2, neutrophil-activating peptide 2; TNF, tumour necrosis factor; RT, reverse transcription; PHA, phytohaemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulphoxide; 1 $\times$ SSC, 0.15 M NaCl plus 0.015 M sodium citrate; poly(A)⁺, polyadenylated; [Ca²⁺]_i, intracellular Ca²⁺ concentration.

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and reverse-transcription PCR (RT-PCR) techniques, we have studied the expression of both IL-8 receptors in myeloid, lymphoid and tissue cells. IL-8R₁ mRNA was found primarily in leucocytes, whereas transcripts for IL-8R₂ (p44) were also detected in non-myeloid cells including lymphocytes and melanoma cells.

EXPERIMENTAL

Cloning of IL-8R₁ cDNA

HL-60 cells at 5×10^5 cells/ml in RPMI 1640 containing 10% fetal-calf serum were differentiated with 1.25% dimethyl sulphoxide (DMSO) and 1 μ M retinoic acid, and their responsiveness to IL-8 was assessed by measuring intracellular Ca²⁺ mobilization (Moser et al., 1991) as a function of culture time (Figure 1). Total RNA was extracted by the guanidine thiocyanate method

Figure 1 Agonist-dependent Ca²⁺ mobilization in HL-60 cells during differentiation

[Ca²⁺]_i changes (percentage Fura-2 saturation) were measured during differentiation of HL-60 cells with 1.25% DMSO and 1 μ M retinoic acid. The cells were stimulated before differentiation (a,b), after 1 day (c,d), 2 days (e,f), 3 days (g,h), 4 days (i,k) and 5 days (l,m) of culture either with 0.1 μ M IL-8 (a,c,e,g,i,l) or 0.1 μ M fMet-Leu-Phe (b,d,f,h,j,k,m). A Fura-2 saturation of 100% refers to the maximum level of Ca²⁺ binding to intracellular Fura-2 as assessed by addition of ionomycin and MnCl₂ (Moser et al., 1991).

(Chirgwin et al., 1979) 12 h before maximum responsiveness (72 h). Purified poly(A)⁺ RNA was reverse-transcribed, and cDNA fragments of more than 1000 bp were cloned into the eukaryotic expression vector, pcDNA-I (Invitrogen Corp.), using *Bsr*XI adaptors as described (Aruffo and Seed, 1987). The resulting cDNA library from differentiated HL-60 cells contained more than 10⁶ individual clones with an average insert size of approx. 1800 bp. For colony-lift hybridization screening (Sambrook et al., 1989), a 819 bp DNA probe, F3R1, was synthesized by PCR (35 cycles, 50 s at 94 °C, 1 min at 60 °C, 1.5 min at 72 °C, buffer conditions as described below) using reverse-transcribed cDNA from rabbit granulocyte poly(A)⁺ RNA and the primers, F3RA and F3RB (Table 1), corresponding to the published cDNA sequence for the rabbit IL-8 receptor (Thomas et al., 1991). Eight clones that strongly cross-hybridized with ³²P-labelled F3R1 were further characterized. Two clones, IL-8R₁171 and IL-8R₁202, conferred specific binding of iodinated IL-8 transfected COS-7 cells, and their cDNAs were sequenced by the dideoxy-sequencing method (Sanger et al., 1977).

Transfection and receptor-binding studies

Jurkat cells that stably expressed IL-8R₁ were obtained by transfection with IL-8R₁202. Briefly, Jurkat cells were washed twice with PBS and incubated on ice for 5 min (5×10^6 – 8×10^6 cells/800 μ l of PBS) with 20 μ g of linearized IL-8R₁202 and 10 μ g of linearized pSV2_{neo}. After electroporation (250 V, 960 μ F, pulse time 30–40 ms) with a gene pulser (Bio-Rad), the cells were placed on ice for 5–10 min, diluted 100-fold with prewarmed RPMI 1640 medium containing 10% fetal-calf serum and cultured for 48 h. The medium was supplemented with G-418 (Gibco/BRL) at 0.8 mg/ml and the culture was continued until the control cells (no pSV2_{neo}) started to die (10–14 days). Clones of neomycin-resistant Jurkat cells were generated by limited dilution, and the cell lines expressing IL-8R₁ were identified by ¹²⁵I-IL-8 binding. IL-8 radioiodination and binding analysis were performed as described by Moser et al. (1991). COS cells were transfected with IL-8R₁171 or IL-8R₁202 by the DEAE-dextran method or by electroporation, and the binding experiments were performed as described (Boulay et al., 1990; Moser et al., 1991).

Table 1 Oligonucleotides for RT-PCR and filter hybridization

The oligonucleotides were synthesized on an Applied Biosystems model 391 automated synthesizer according to standard protocols. Orientation of the primers refers to hybridization to either the sense or antisense template DNA. The nucleotide sequences of the template DNAs are indicated under specificity. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Primers	Orientation	Specificity	Size of RT-PCR products (bp)
SE1-R ₁	5'-ACATGGAGAGTGACAGCTTTGAAG-3'	Sense	
AS1-R ₁	5'-GCCAACAAGGAAGGCTGCTGTC-3'	Antisense	
SE2-R ₁	5'-CAGTTACAGCTCTACCTGCC-3'	Sense	IL-8R ₁ (see Figure 3)
AS2-R ₁	5'-GCTGTCTTTGGGCCAGGAGTC-3'	Antisense	IL-8R ₁ (see Figure 3)
SE1-R ₂	5'-AGATCCACAGATGTGGGATTTGA-3'	Sense	
AS1-R ₂	5'-CAGTTACAGCTCTACCTGCC-3'	Antisense	IL-8R ₂ (Holmes et al., 1991)
SE2-R ₂	5'-CGCCAGGCTTACCATCCAACA-3'	Sense	
AS2-R ₂	5'-GGTAACACGATGACGTGCCAAG-3'	Antisense	IL-8R ₂ (Holmes et al., 1991)
SE-GAP	5'-ACATCAAGAAGGTGGTGAAGCAGG-3'	Sense	
AS-GAP	5'-CTCTTCTCTTGTGCTCTTGTGG-3'	Antisense	GAPDH (Tokunaga et al., 1987)
F3RA	5'-CTGTAGAAAAAGATTATAGCCCTGTCTGGTA-3'	Sense	
F3RB	5'-CTGCCGTGGCTCTAAGACCCGAAG-3'	Antisense	Rabbit IL-8R (Thomas et al., 1991)
IN1-R ₁	5'-AAGGACCGTCTACTCATC-3'	Probe for IL-8R ₁ (see Figure 3)	
IN1-R ₂	5'-TTTGGATGGTAAGCCTGG-3'	Probe for IL-8R ₂ (see Figure 3)	
IN-GAP	5'-GCAACAGGGTGGTGGACC-3'	Probe for GAPDH (Tokunaga et al., 1987)	

Northern-blot analyses

RNA from human blood leucocytes, lymphocytes, leukaemic cell lines, cultured tissue cells and melanomas was extracted by the guanidine thiocyanate method (Chirgwin et al., 1979) and stored at -70°C in 99% ethanol. Before Northern blotting, each RNA sample was analysed by formamide/formaldehyde/agarose-gel electrophoresis with ethidium bromide staining (Sambrook et al., 1989). Routinely, 10 μg RNA samples were separated on formamide/formaldehyde/agarose gels, vacuum-transferred (trans-Vac TE 80, Hoefer Scientific Instruments) on to Nytran membranes (Schleicher & Schuell), and immobilized by baking at 80°C for 90 min. The same procedure was performed with RNA from blood neutrophils (2 μg) and poly(A)⁺ RNA from retinoic acid/DMSO-differentiated HL-60 cells (3 μg). The hybridization probes for IL-8R₁, a 562 bp *Bgl*III–*Bst*XI fragment of IL-8R₁, 202, and β -actin (Gunning et al., 1983) were ³²P-labelled by random priming to $> 2 \times 10^9$ d.p.m./ μg . The membranes were prehybridized and hybridized ($> 10^6$ c.p.m. probe/ml) (Sambrook et al., 1989), washed in $0.5 \times \text{SSC}$ containing 0.1% SDS at 68°C , and exposed to Hyperfilm-MP (Amersham International plc).

cDNA synthesis and PCR amplification

Total RNA from various cell sources (2 μg , except for 1 μg of RNA from human blood neutrophils) was mixed with 0.9 μg of oligo-dT₁₅ in a total volume of 5 μl , heated for 5 min at 70°C and kept at room temperature for 10 min. To start cDNA synthesis, PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin) supplemented with MgCl₂ to give 6 mM, 1 mM dNTPs, 30 units of rRNasin (Promega Corporation) and 24 units of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim) were added and the 20 μl reaction mixtures were incubated at 37°C for 60 min. Subsequently, 30 μl of water was added, and the reactions were terminated by heating for 5 min at 95°C . PCR-amplification mixtures (50 μl) contained 5 μl of the cDNA preparations, PCR buffer, 0.4 μM primers and 280 μM dNTPs and were capped with paraffin oil. After denaturation (2.5 min at 96°C) and 'hot start' [additions of 1 unit of AmpliTaq polymerase (Perkin-Elmer Cetus) at 85°C] (Frohman et al., 1988), cDNAs were amplified for 25 cycles [1 min at 55°C for IL-8R₁ cDNA primers, at 53°C for IL-8R₂ cDNA primers or at 60°C for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA primers, 1.5 min at 72°C and 0.5 min at 96°C] in a Techne PHC-2 thermal cycler (Brouwer AG). All components of the amplification mixtures were tested for contaminants by 35 PCR cycles in the absence of cDNA template. Contamination of RNA preparations with genomic DNA was assessed by reverse transcription in the absence of AMV reverse transcriptase followed by PCR as described above. The specific primers for IL-8R₁ cDNA were SE1-R₁ and AS1-R₁, and those for IL-8R₂ cDNA were SE1-R₂ and AS1-R₂ (Table 1). The primers for GAPDH, i.e. SE-GAP and AS-GAP, were chosen to span a cDNA fragment of 282 bp and, in case of genomic DNA contamination due to an intron, a DNA fragment of 386 bp. Amplified products were analysed by ethidium bromide staining on agarose gels and Southern hybridization. Samples (10 μl) of the PCR reaction mixtures were resolved on 1% (w/v) agarose gels, denatured, neutralized and transferred to Nytran membranes as described (Sambrook et al., 1989). The membranes were hybridized with the ³²P-labelled probes, IN1-R₁, IN1-R₂ and IN-GAP, at 42°C in a solution containing $6 \times \text{SSC}$, $5 \times \text{Denhardt's}$, 100 $\mu\text{g}/\text{ml}$ denatured calf thymus DNA and 0.5% SDS (10^6 c.p.m. of labelled probe/ml).

Before autoradiography, the membranes were washed in $0.5 \times \text{SSC}$ plus 0.1% SDS at 42°C .

The cDNA samples were also analysed by the PCR method with different sets of primers under different conditions in a second laboratory (Milano). In addition, 1 μg samples of independently isolated RNA from melanomas, monocytes, neutrophils, lymphocytes, leukaemia cell lines and cultured melanocytes were reverse-transcribed (Moloney murine leukaemia virus reverse transcriptase; BRL, Life Technologies) with oligo-dT₁₅ in 30 μl volumes, as described above, to test by PCR. The amplifications were carried out in 25 μl reaction volumes containing 1 μM primers, 0.6 unit of AmpliTaq, 1.5 mM dNTPs and $1 \times \text{PCR}$ buffer for 30–40 cycles (1 min at 94°C , 2 min at 60°C and 3 min at 72°C). Primers for IL-8R₁ were SE2-R₁ and AS2-R₁, and those used for IL-8R₂ were SE2-R₂ and AS2-R₂ (Table 1). Routinely, mapping of restriction-enzyme sites (*Nde*I for IL-8R₁ DNA and *Hpa*II for IL-8R₂ DNA) by agarose-gel electrophoretic analysis was employed for verification of the PCR products.

Cell culture

Monocytes and neutrophils were isolated from human blood (Peveri et al., 1988; Walz et al., 1991a). Melanocytes, melanomas from human biopsies and the CD4⁺ T-cell clone were maintained as described (Radrizzani et al., 1991; Colombo et al., 1992). Phytohaemagglutinin (PHA)-activated T-lymphocyte blasts were kindly prepared by Dr. P. Della Bona, Basel Institute of Immunology. All established cell lines were cultured according to standard conditions.

RESULTS

IL-8 receptor expression in HL-60 cells

The responsiveness of HL-60 cells to IL-8 was assessed by real-time measurements of changes in intracellular Ca²⁺ concentration [Ca²⁺]_i. Undifferentiated cells regularly showed small but detectable [Ca²⁺]_i transients (Figure 1). Addition to the culture medium of DMSO or retinoic acid alone (not shown), or in combination, led to a marked increase in the intensity of the response which reached maximum levels after 3–4 days. Responsiveness to the unrelated chemotactic peptide, fMet-Leu-Phe, was enhanced to similar extents. Differentiation for more than 4 days led to a change in the time course of the [Ca²⁺]_i response characterized by a slower return to background levels. An increase in IL-8-induced [Ca²⁺]_i mobilization indicated that differentiation of HL-60 cells with DMSO and/or retinoic acid was paralleled by an increased expression of receptors for IL-8.

For the cloning of IL-8 receptor cDNAs, HL-60 cells were treated for 60 h with DMSO and retinoic acid, RNA was extracted and a cDNA library was constructed (see the Experimental section). Transfection of COS cells with two clones that were isolated, IL-8R₁171 and IL-8R₁202, resulted in specific but low-affinity binding of IL-8 ($K_d > 24$ nM; not shown). However, stable transfectant Jurkat cells, expressing 17000 ± 1000 receptors per cell, bound IL-8 with high-affinity (K_d 1.0 nM) similar to neutrophils (Figure 2). In addition, the transfectants bound GRO α (K_d 0.5 nM) and NAP-2 (K_d 0.7 nM, not shown) with high affinity.

In IL-8R₁171 and IL-8R₁202 the regions coding for a polypeptide of 360 amino acids with seven putative transmembrane domains and the 3'-untranslated sequences are identical with those of the IL-8R₁ receptor cDNA described by Murphy and Tiffany (1991). Interestingly, the sequences diverged at position –25 in the 5'-untranslated region (Figure 3). Whereas in IL-

Figure 2 Binding of ^{125}I -IL-8 and ^{125}I -GRO α to transfected Jurkat cells

Binding studies to Jurkat cells stably expressing IL-8R $_1$ and analysis of the binding data were performed as described (Moser et al., 1991; Schumacher et al., 1992). The cells were incubated with ^{125}I -IL-8 (a) or ^{125}I -GRO α (c) in the presence (▲) or absence (●) of 1 μM unlabelled IL-8 or GRO α to determine the specific binding (●). Scatchard transformations for IL-8 and GRO α binding are shown in (b) and (d) respectively. Binding of 1 nM corresponds to 40 fmol of IL-8 (b) or 60 fmol of GRO α (d) bound to 10^6 cells and 1 unit of bound/free equals 40 μl (b) or 60 μl (d) per 10^6 cells.

8R $_1$ 202 a sequence of 307 nucleotides preceded the junction of divergence (position -25), IL-8R $_1$ 171 contained a unique short stretch of 39 nucleotides. Partial sequence analysis of the 5'-regions of a number of additional clones indicated that IL-8R $_1$ 202 corresponded to the predominant IL-8R $_1$ RNA species in differentiated HL-60 cells. RNA corresponding to IL-8R $_1$ 171 did not appear to be highly expressed in HL-60 cells, suggesting that this clone may have arisen from alternative splicing events (Ueda et al., 1987). In this regard it is interesting to note that all cDNAs analysed in the present study (except for IL-8R $_1$ 171) had unusually long 5'-untranslated sequences (> 320 nucleotides), which is in striking contrast with the recently published cDNAs (Murphy and Tiffany, 1991; Holmes et al., 1991).

IL-8 receptor expression in blood cells

IL-8R $_1$ -specific transcripts were of different length and were highly enriched in the total RNA extracted from human neutrophils (Figure 4). With the internal *Bgl*II-*Bst*XI fragment (Figure 3) as hybridization probe, three major transcripts of 2.2, 2.8 and 3.2 kb and a minor one of > 7.5 kb were detected by Northern-blot analysis. By contrast, only a single RNA band of approx. 3 kb was found in poly(A) $^+$ RNA from differentiated HL-60 cells. The expression of this transcript was induced in HL-60 cells by DMSO and retinoic acid alone or in combination, but remained well below the level seen in RNA from neutrophils.

Figure 3 Complete nucleotide sequences of the two IL-8 receptor cDNA clones, IL-8R $_1$ 171 and IL-8R $_1$ 202

Asterisks indicate the nucleotides that are identical in the coding sequence of IL-8R $_1$ and IL-8R $_2$ (Holmes et al., 1991). The positions of the PCR primers, SE1-R $_1$, SE2-R $_1$, AS1-R $_1$, AS2-R $_1$ and the oligonucleotide, IN1-R $_1$, used for Southern hybridizations are underlined. The sites for the restriction endonucleases, *Bgl*II and *Bst*XI, used to prepare the DNA probe for Northern-blot analysis are also indicated. The restriction endonucleases, *Nde*I and *Hpa*II, were used for verification of the RT-PCR products. Note that the IL-8R $_1$ -specific PCR product contains, in addition to the *Hpa*II site that is underlined, a second *Hpa*II site at position 720.

Figure 4 Northern-blot analysis of neutrophils and differentiated HL-60 cells

(a) Total RNA from human blood neutrophils (2 μ g), (b) poly(A)⁺ RNA from HL-60 cells differentiated with DMSO plus retinoic acid (3 μ g), and total RNA (10 μ g) from (c) undifferentiated, (d) DMSO-differentiated, (e) retinoic acid-differentiated and (f) DMSO plus retinoic acid-differentiated HL-60 cells. For differentiation, HL-60 cells were kept in culture for 60 h in the presence of 1.25% DMSO and/or 1 μ M retinoic acid. The ³²P-labelled *Bgl*II–*Bst*XI fragment from IL-8R₁ was used as hybridization probe.

RNA from myelocytic (KG-1), erythroleukaemic (K-562) and monocytic (THP-1, U 937, AML 193) cell lines, human monocytes, Jurkat cells and PHA-activated T-cell blasts was analysed by the same technique, but hybridization was negative, except for AML 193 and U 937 where a faint band was detected (results not shown).

The coding nucleotide sequences of the IL-8R₁ and IL-8R₂ cDNA show a high degree of identity; in particular, the regions spanning the sequences of the first and seventh putative transmembrane domains (positions 142–951 in Figure 3) are 89% identical without the introduction of a single gap. Since the probe used for Northern-blot analysis overlapped considerably with this region, hybridization with the transcripts for both IL-8R₁ and IL-8R₂ must have occurred. To selectively assess expression of the two receptors, RT-PCR analysis was therefore performed using primers corresponding to less conserved regions at both ends of the coding sequences. RNAs were reverse-transcribed and amplified using the IL-8R₁-specific primers, SE1-R₁ and AS1-R₁ (Figure 3). PCR conditions were such that cDNA amplification, except for neutrophil-derived cDNA, did not reach stationary phase. The products of 1037 bp were identified with the ³²P-labelled internal probe, IN1-R₁, by Southern hybridization (Figure 5). As expected, differentiated and undifferentiated HL-60 cells expressed IL-8R₁ mRNA, but the levels remained below those observed in neutrophils, in agreement with the results of Northern-blot analysis (Figure 4). IL-8R₁ mRNA transcripts were also detectable in human blood mononuclear cells and the monocytic cell line, AML 193, but not in THP-1 and Jurkat cells, and in PHA-activated T-cell blasts (see below). The cellular expression of IL-8R₂ transcripts was studied with PCR primers at positions corresponding to those of the IL-8R₁ primers, spanning a fragment of 1042 bp. PCR conditions were identical, except for the annealing temperature that was lowered by 2 °C to adjust for small differences in the

Figure 5 IL-8R₁ and IL-8R₂ transcript analysis in blood cells by RT-PCR

Samples of total RNA from cells as indicated were reverse-transcribed, amplified by PCR and analysed after agarose-gel separation and transferred to a nylon membrane. PCR primers for IL-8R₁, IL-8R₂ and GAPDH were SE1-R₁/AS1-R₁, SE1-R₂/AS1-R₂ and SE-GAP/AS-GAP respectively. Hybridizations were performed with the ³²P-labelled oligonucleotide probes, IN1-R₁, IN1-R₂ or IN-GAP, and the membranes were washed as described in the Experimental section and exposed to autoradiography film for 16 h.

melting temperature of the primers. As shown in Figure 5, IL-8R₂ mRNA was expressed in neutrophils, monocytes and DMSO-treated HL-60 cells. Transcripts for IL-8R₂, however, were also found in all other samples tested, including RNAs from Jurkat cells and PHA-activated T-lymphoblasts, when the amplification was extended to 30 cycles (results not shown). The efficiency of reverse transcription was assessed by amplification of GAPDH cDNA.

To estimate the sensitivity of IL-8R₁-specific RT-PCR, the cDNA of IL-8R₁171 was serially diluted using graduated capillary tubes, and amplified under the same conditions as for the analysis of cellular RNA samples. The membranes containing the immobilized PCR products were hybridized with ³²P-IN-R₁ in the same sealing bag as the example shown in Figure 5 and autoradiography was performed for the same period of time. An input of less than 7530 IL-8R₁171 fragments (0.0125 amol) per PCR reaction (dilution 5 in Figure 6) resulted in linear amplification, and the sensitivity limit was around 400 molecules per reaction. Thus, for instance, the signal obtained with RNA extracted from undifferentiated HL-60 cells corresponded to approx. 2500 IL-8R₁-specific cDNA molecules per reaction or 12500 cDNA molecules per μ g of total RNA.

These RT-PCR experiments were repeated using the same as well as newly isolated RNA samples, with other sets of primers (SE2-R₁, AS2-R₁ and SE2-R₂, AS2-R₂, see Table 1) under different amplification conditions. The PCR products (964 bp for IL-8R₁ and 489 bp for IL-8R₂) were analysed by agarose-gel electrophoresis and identified by restriction-enzyme analysis, and the efficiency of reverse transcription was assessed with primers for β -actin or GAPDH (not shown). In agreement with the results presented in Figure 5, IL-8R₁-specific transcripts were

Figure 6 Determination of the range of exponential PCR amplification by serial dilution of an IL-8R₁ cDNA fragment

IL-8R₁171 cDNA was diluted in 2-fold steps in the range 0.2–0.0002 amol per PCR reaction (dilutions 1 to 11) in 1 × PCR buffer containing 1 µg/ml heat-denatured salmon sperm DNA and amplified using the primers, SE1-R₁ and AS1-R₁. Amplification products were analysed by agarose-gel electrophoresis and ethidium bromide staining (c), transferred on to a nylon membrane, hybridized with ³²P-labelled IN1-R₁, washed and exposed to autoradiography film (b) exactly as shown in Figure 5. The exposed film was scanned using a densitometer and the relative densitometry units were plotted against the serial dilution of the IL-8R₁171 cDNA (a). The straight line denotes the slope in the exponential phase of PCR amplification.

detected exclusively in neutrophils, monocytes, HL-60 and AML 193 cells, confirming that IL-8R₁ expression is restricted to myeloid cells (Figure 7). A much wider distribution was found for IL-8R₂ transcripts which were clearly detectable in all myeloid cells as well as in PHA-activated T-lymphocyte blasts, a CD4⁺ T-cell clone and, to a lesser extent, Jurkat cells, again confirming the results of the first series of RT-PCR assays. The limit of detection using the second set of IL-8R₁ primers was assessed with a serial dilution of IL-8R₁171 cDNA fragments as outlined above. Approx. 4-fold higher amounts of cDNA fragments were required for amplification signals comparable with those in Figure 6 (not shown). In addition, the primers SE2-R₂/AS2-R₂, as used in Figure 7, were found to be more than 10-fold more sensitive than the former primer set (Figure 5). Therefore, the subsequent PCR studies were carried out with the primer sets, SE1-R₁/AS1-R₁ and SE2-R₂/AS2-R₂, under the conditions described in the Experimental section.

IL-8 receptor expression in melanoma cells

It has been proposed repeatedly that IL-8 and GRO α may regulate the migration and growth of tissue cells. Of particular

Figure 7 Analysis of transcripts for IL-8R₁ and IL-8R₂ in leucocytes and lymphocytes

Samples of total RNA were reverse-transcribed and amplified by PCR for 40 cycles using the primer sets, SE2-R₁/AS2-R₁ and SE2-R₂/AS2-R₂. Differentiation of HL-60 cells with DMSO and retinoic acid is described in Figure 4. The RT-PCR products were separated on agarose gels and visualized by ethidium bromide staining. As a control for the purity of the PCR reagents, amplification was performed without addition of cDNA. The DNA size markers were ϕ X174 cut with *Hae*III.

Figure 8 Expression of IL-8R₁ and IL-8R₂ mRNA in human melanoma cells, melanocytes and fibroblasts

RNA from 19 primary melanoma cell cultures, melanocytes and fibroblasts were reverse-transcribed. The cDNA samples were amplified for 35 cycles with the PCR primer sets, SE1-R₁/AS1-R₁ and SE2-R₂/AS2-R₂, under the conditions described in Figure 7. The amplification products were analysed by agarose-gel electrophoresis and ethidium bromide staining. The results of a representative selection of six melanomas are shown in this Figure. The RT-PCR control (no cDNA) and the DNA size markers (ϕ X174) were as in Figure 7. As a positive control for the PCR reactions, cDNA from DMSO-differentiated HL-60 cells was amplified and analysed.

interest are melanoma cells since they were reported to proliferate in response to GRO α (Richmond et al., 1988) and to migrate in response to IL-8 (Wang et al., 1990). Interestingly, melanoma

cell lines constitutively produce considerable amounts of IL-8 (Colombo et al., 1992) and GRO α (M. Colombo, unpublished work), which could act in auto- or para-crime fashion (Murphy and Tiffany, 1991; Holmes et al., 1991). RT-PCR analysis showed a clear-cut difference in the expression of the two IL-8 receptors. All 19 cell lines established from human primary melanomas or metastases (a selection of which is shown in Figure 8), both melanocyte cell lines and the fibroblasts expressed transcripts for IL-8R $_2$. As shown in Figure 8, however, all these cells, with the single exception of one of the melanocyte lines, were negative for IL-8R $_1$ transcripts.

DISCUSSION

Human neutrophils contain two high-affinity receptors for IL-8 (Moser et al., 1991; Schnitzel et al., 1991; Schumacher et al., 1992) which can be distinguished by their electrophoretic mobility (p44 and p70) and by the binding of GRO α or NAP-2, which is of high affinity for p70 and low affinity for p44 (Schumacher et al., 1992). The present results show that transcripts for two IL-8 receptors that were recently cloned, IL-8R $_1$ (Murphy and Tiffany, 1991) and IL-8R $_2$ (Holmes et al., 1991), are abundantly expressed in human neutrophils, and it is reasonable to assume that the two cDNAs code for the receptors that have been identified biochemically (Moser et al., 1991; Schnitzel et al., 1991; Schumacher et al., 1992). In contrast with COS cells, expression of IL-8R $_1$ in Jurkat cells resulted in high-affinity binding of IL-8 as well as GRO α and NAP-2. Although the lack of protein-sequence information precludes unequivocal assignment, these binding data indicate that IL-8R $_1$ corresponds to p70 in neutrophils, whereas p44 appears to be encoded by IL-8R $_2$.

The occurrence of two classes of IL-8 receptors in human neutrophils is unique, as only single-receptor cDNAs have been found so far for several other chemotactic agonists, such as fMet-Leu-Phe (Boulay et al., 1990), C5a (Gerard and Gerard, 1991; Boulay et al., 1991) and platelet-activating factor (Nakamura et al., 1991). It is also noteworthy that the IL-8 analogues, GRO α and NAP-2, share these receptors with IL-8, but bind with lower affinity with receptors of one class (Schumacher et al., 1992). The two receptors could signal different neutrophil responses, and the low-affinity interaction of GRO α and NAP-2 with one of them may explain why GRO α and NAP-2 are considerably less potent than IL-8 as inducers of exocytosis (Walz et al., 1989; Moser et al., 1990; Clark-Lewis et al., 1991) and the respiratory burst (Moser et al., 1990; Walz et al., 1991a). It is conceivable, however, that the responses elicited by IL-8 and its two analogues may solely be dependent on high-affinity binding, and that the low-affinity receptors for GRO α and NAP-2 are associated with other, as yet unknown, functions that may be studied in cells transfected with single-receptor proteins.

There is a striking difference in the cellular distribution of IL-8R $_1$ and IL-8R $_2$. IL-8R $_1$ expression is restricted to neutrophils, monocytes and a few myeloid cell lines, suggesting again that the main function of this receptor is related to neutrophil activation and the mobilization of phagocytes in host defence and inflammation. In view of the wide distribution of IL-8R $_2$, which is strongly expressed in myeloid cells but also in lymphocytes, melanoma cells, melanocytes and fibroblasts, it can be assumed that this receptor may mediate migration and growth in cells that are not involved in host defence. It must be kept in mind, however, that the highest levels of IL-8R $_2$ transcripts were found in neutrophils, and the possibility should be considered that activation of this receptor may elicit neutrophil responses that are not related to chemotaxis and release activities.

The potential effects of IL-8 on non-myeloid cells have recently gained considerable attention. Of particular interest were the observations that GRO α stimulates melanocyte growth (Richmond et al., 1988) and IL-8 induces melanoma cell migration (Wang et al., 1990). Analysis of a large number of cell lines established from primary melanomas or metastases showed that these cells in most instances constitutively produce large amounts of IL-8 and GRO α (Colombo et al., 1992; M. Colombo, unpublished work). Both cytokines could exert auto- or para-crime growth and/or migration effects on the tumour cells (Wang et al., 1990; Richmond et al., 1988). These effects are likely to be mediated by IL-8R $_2$, which was shown in this study to be expressed by all tumour cells. Further studies are needed to assess the potential competition or synergistic action of IL-8 and GRO α on melanoma cells, and to find out whether the constitutive production and release of these cytokines leads to receptor desensitization. The significance of the expression of IL-8R $_1$ in one of two melanocyte cell lines tested is unclear. The presence of this receptor could be a characteristic of an IL-8-responsive phenotype, as formerly reported for interleukin 6 in melanoma cell cultures (Lu and Kerbel, 1992).

The fact that human T-lymphocytes express IL-8R $_2$ but not IL-8R $_1$ suggests that the reported effect of IL-8 on lymphocyte migration may depend on IL-8R $_2$ (Larsen et al., 1989). Binding studies, however, have revealed very low numbers of IL-8 receptors in lymphocytes, and it may be questioned whether the presence of IL-8R $_2$ mRNA, as detected by the present study, is actually sufficient for the expression of functional receptors in melanomas. During the preparation of this paper, a report by Michel et al. (1992) appeared showing that normal human keratinocytes which were chemotactically attracted by IL-8 also contained mRNA for IL-8R $_2$. This observation is in agreement with the present demonstration that expression of IL-8R $_2$, in contrast with IL-8R $_1$, is not restricted to blood leucocytes.

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