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Chemokine production by human vascular smooth muscle cells: modulation by IL-13

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1 The production of chemokines by vascular smooth muscle cells (SMC) is implicated in the pathogenesis of atherosclerosis, although the factors regulating chemokine production by these cells are incompletely characterized.

2 We describe the differential stimulation of interleukin-(IL)-8, monocyte chemoattractant protein (MCP)-1 and regulated on activation normal T-cell expressed and secreted (RANTES) synthesis following treatment of human vascular SMC with IL-1α or tumour necrosis factor α (TNFα). Under basal conditions, cultured SMC release very low amounts of IL-8, MCP-1 and RANTES as assessed by specific ELISA. Concentration-response studies with IL-1α or TNFα revealed that each stimulus induced a similar amount of MCP-1. In contrast approximately three fold more IL-8 was induced by IL-1α than by TNFα whereas significant RANTES production was induced only by TNFα. These findings point to a divergence in the regulation of synthesis of the different chemokines in response to IL-1α or TNFα stimulation.

3 The T-cell derived cytokines IL-10 and IL-13 were also found to have differential effects on chemokine production by SMC. IL-13, but not IL-10, significantly enhanced IL-8 and MCP-1 release in response to IL-1α or TNFα. This increase in chemokine release appeared to be accounted for by increased mRNA expression.

4 These findings provide support for the concept that smooth muscle cells can have an active role in a local immune response via the production of chemokines which can be selectively modulated by T-cell derived cytokines.

Keywords: Chemokine; interleukin (IL)-1α; IL-8; IL-10; IL-13; TNFα; RANTES; MCP-1; smooth muscle

Introduction

The characteristic fatty streaks and fibrofatty lesions of atherosclerosis result from the accumulation of lipid laden macrophages, lymphocytes and smooth muscle cells (SMC) in the tunica intima of the vessel wall (Raines & Ross, 1993). The migration and activation of these specific cell types probably occurs in response to the production of selective chemoattractants by inflammatory cells, as well as by the structural cells present in the vessel wall, including the smooth muscle and endothelial cells.

The chemokines are a superfamily of structurally related 8–10 kDa chemotatic proteins originally identified for their ability to attract selectively distinct populations of leukocytes. The superfamily can now be divided into three sub-families dependent upon the number and position of the cysteine residues (Kunkel *et al*., 1995). The C-X-C family, of which the prototype interleukin-8 (IL-8) is a member, is characterized by having four cysteine residues, the first two are separated by a single amino acid. Their target cells include neutrophils (Yoshimura *et al*., 1987), T-lymphocytes (Larsen *et al*., 1989; Bacon *et al*., 1989) and microvascular endothelial cells (Koch *et al*., 1992). The C-C family has four cysteines in which the first two are adjacent, members include monocyte chemoattractant protein-1 (MCP-1) and RANTES. MCP-1 induces chemotaxis of monocytes (Yoshimura *et al*., 1989) and lymphocytes (Taub *et al*., 1995; Roth *et al*., 1995) and induces histamine release in basophils (Kuna *et al*., 1993), while RANTES induces chemotaxis of memory T-cells (Schall *et al*., 1990; Turner *et al*., 1995; 1996; Murphy *et al*., 1994), monocytes (Wiedermann *et al*., 1993) and eosinophils (Kameyoshi *et al*., 1992). The C chemokine family to date has only one member, the lymphocyte chemoattractant lymphotactin which has in total two cysteines (Kelner *et al*., 1994).

There is a large body of evidence indicating that chemokines are involved in the pathogenesis of atherosclerosis. Monocyte chemoattractant protein (MCP)-1 is expressed in macrophage rich areas of human and rabbit atherosclerotic lesions (Ylä-Herttuala *et al*., 1991; Takeya *et al*., 1993), in the vessels of hypercholesterolaemic primates (Yu *et al*., 1992) and in the coronary vessels of experimental cardiac transplants (Russell *et al*., 1993). In addition, vascular SMC in culture when stimulated with modified low density lipoprotein (LDL), platelet derived growth factor (PDGF), thrombin or the proinflammatory cytokines interleukin-1α (IL-1α) or tumour necrosis factor α (TNFα), express MCP-1 (Cushing *et al*., 1990; Wang *et al*., 1991; Taubman *et al*., 1992; Grandaliano *et al*., 1994; Lukacs *et al*., 1995). Although IL-1α and TNFα are present in atherosclerotic lesions (Raines & Ross, 1993) and are good stimuli for MCP-1 generation, their effect on the quantal and temporal expression of IL-8 and RANTES production by vascular SMC has not been determined.

The production of chemokines by activated vascular SMC may be modulated by products of activated T-lymphocytes with which they have a close association in atherosclerotic lesions (Stemme & Hansson, 1994). The T-lymphocyte derived cytokines IL-10 and IL-13 are potent modulators of immune function (Howard *et al*., 1992; Minty *et al*., 1993) and their ability to inhibit secretion of pro-inflammatory cytokines and the chemokines IL-8 and macrophage inflammatory protein-1α (MIP-1α) from lipopolysaccharide (LPS) stimulated human monocytes, has led to them being termed anti-inflammatory cytokines (De Waal Malefyt *et al*., 1993; Moore *et al*., 1993). However, little is currently known about the effects of IL-10

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and IL-13 on human vascular SMC. We show here that in human SMC IL-13, but not IL-10, selectively upregulates chemokine generation.

Methods

Culture of human smooth muscle cells

Human vascular SMC were obtained from 5 saphenous vein specimens and 1 aortic specimen by use of either an explant or collagenase/trypsin/EDTA digestion method essentially as previously described (Chamley-Campbell *et al*., 1979). The vessel was opened longitudinally and scraped to remove endothelial cells. In the explant method small (6 mm^2) moistened pieces of tissue were placed intima-side down in a tissue culture flask. The tissue was left to adhere for 2 h, then the base of the flask was carefully flooded with culture medium and left undisturbed for 2–3 weeks. The culture medium used for SMC was Dulbecco's modified Eagle's medium (DMEM) containing 15% HI-FBS, penicillin (10 u ml^{-1}), streptomycin (10 $\mu g\ ml^{-1}$) and fungizone (0.5 $\mu g\ ml^{-1}$), this will be called 'complete DMEM'. In the digestion method minced vessel was shaken at 37°C for 2 h with collagenase (1 mg ml^{-1}), followed by 3×10 min with trypsin/EDTA. The washed single cells were placed in a flask and left for 1–2 weeks in complete DMEM. When the primary isolates had grown to confluence, cells which had initially been obtained in different ways, were cultured identically in complete DMEM. Once the cells had been subcultured twice with trypsin/EDTA, the cultures were characterized immunohistochemically. Cells were fixed for 10 min in ice-cold methanol and all cultures were shown to be positive for SMC specific α-actin by an anti-α actin fluorescein isothiocyanate conjugated antibody (Sigma). There were no apparent differences in the morphology or staining between the aortic and the venous cultures, nor between the cells prepared with the explant or digestion method of isolation. Initial experiments were performed with cells that had been isolated by the digest method. However, we later found that the explant method resulted in better initial cell growth. All experiments were carried out with 3rd to 10th passage (p3–10) cells that had been rendered quiescent by culture for 24 h (or 48 h where indicated in figure legends) in serum-free medium. Each experiment was repeated with cells obtained from at least 3 different donors.

Measurement of secreted IL-8, MCP-1 and RANTES protein by enzyme-linked immunosorbent assay (ELISA)

Smooth muscle cells were grown to confluence in 24 well plates (Linbro) coated with 1% gelatin (Sigma), this was found to enhance cell adhesion and increase the growth rate. Following treatment with IL-1α or TNFα alone or in combination with IL-10 or IL-13, cell culture supernatants were removed and the chemokine content was measured by ELISA. Cell viability was determined by examination of cell morphology and monolayer integrity at the conclusion of each experiment.

MCP-1 protein was measured in a sandwich ELISA as previously described (Yoshimura *et al*., 1991). Briefly, microtitre plates (Maxisorb, Nunc) were coated overnight with monoclonal MCP-1 antibody (4.5 $\mu g\ ml^{-1}$ in sodium bicarbonate buffer, pH 9.6). The plates were blocked for 1 h at 37°C with 0.2% bovine serum albumin in Tris-buffered saline (TBS, 42 mM Tris, 150 mM NaCl pH 7.3). Samples and standards in TBS + 0.05% Tween 20 were added for 90 min at 37°C, the standard curve range was from 0.25–8 ng ml^{-1}. Plates were washed and incubated with polyclonal rabbit anti-MCP-1 (90 min 37°C). Binding was detected by use of alkaline phosphatase labelled anti-rabbit IgG followed by *p*-nitrophenylphosphate as the substrate.

Secreted IL-8 and RANTES protein were measured by sandwich ELISA. Microtitre plates were coated overnight at 4°C with monoclonal anti-IL-8 or RANTES antibody (1 $\mu g\ ml^{-1}$ in sodium bicarbonate buffer, pH 9.6). Plates were washed 3 times with PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) + 0.05% Tween 20 and incubated for 2 h at 37°C with samples or standards in PBS + 2% FCS + 0.05% Tween 20. The detecting antibody was biotin conjugated polyclonal goat IgG anti-IL-8 or RANTES added at 1 $\mu g\ ml^{-1}$ for 1 h at 37°C. ELISAs were developed by incubation with streptavidin horseradish peroxidase (1 $\mu g\ ml^{-1}$ for 15 min) followed by *o*-phenylenediamine dihydrochloride and H_2O_2 as the substrate. The standard curve of the assays ranged from 0.2–4 ng ml^{-1}. For each independent experiment the mean chemokine secretion was determined from two wells of culture supernatant, each measured in duplicate. Replicate experiments were performed as detailed in figure legends.

Northern blot analysis

Total RNA was extracted from SMC grown in 25 cm^2 flasks by use of RNAzol, a commercial preparation of guanidinium thiocyanate (Tel Test, Texas, U.S.A.). RNA was precipitated, dissolved in water and the concentration measured by absorbance at 260 nm. Approximately 5 μg total RNA was loaded per lane of a 1% agarose/formaldehyde gel. Ethidium bromide was included in each sample enabling equal loading to be assessed by observation of the 18S and 28S ribosomal RNA. RNA was transferred overnight to nylon membrane (Boehringer Mannheim) by capillary blotting and fixed by baking at 120°C for 20 min.

The hybridization protocol was essentially as described in the digoxigenin (DIG) luminescent detection kit (Boehringer Mannheim). Briefly, membranes were prehybridized for 1 h at 42°C then hybridized overnight at the same temperature with DIG-labelled oligonucleotide probes (10 ng ml^{-1}). Unbound probe was removed by washing in $2 \times$ SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS (2×5 min) followed by $0.1 \times$ SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0), 0.1% SDS (2×10 min). Bound probes were detected by use of anti-DIG Fab conjugated to alkaline phosphatase followed by a chemiluminescent substrate (CSPD) (Boehringer Mannheim). Bands were visualized by exposure to X-ray film for 1–2 h and quantified by scanning densitometry. Results were corrected for differences in loading according to the β-actin control, as described in the figure legends.

Cytokines and reagents

Human recombinant IL-1α (specific activity 5×10^7 u mg^{-1}) and TNFα (specific activity 6×10^7 u mg^{-1}) were generous gifts from Glaxo (Greenford, U.K.) and Bayer (Slough, U.K.), respectively. Human recombinant IL-13 was a gift from Dr Adrian Minty (Sanofi Research, Labege, France). Human recombinant IL-10 (specific activity 1×10^7 u mg^{-1}) was a gift from Dr K.W. Moore (DNAX, Palo Alto, California, U.S.A.). All cell culture reagents and heat inactivated foetal bovine serum (HI-FBS) were from Gibco BRL (Paisley, Scotland). Linbro 24 well tissue culture plates were from ICN (Thame, U.K.). The digoxigenin (DIG) chemiluminescent detection kit for Northern blotting was from Boehringer Mannheim (Lewes, U.K.). 5′Digoxigenin labelled probes for IL-8, MCP-1, regulated on activation normal T-cell expressed and secreted (RANTES) and β-actin mRNA were cocktails of 30-mer oligonucleotides (R&D Systems, Abingdon, U.K.). Antibodies for the IL-8 and RANTES enzyme linked immunosorbent assays (ELISAS) were purchased from R&D Systems. Chemokine standards for ELISA were from National Institutes for Biological Standards and Control (Hertfordshire, U.K.). All other chemicals were from Sigma (Poole, U.K.) or Fisons (Loughborough, U.K.).

Statistics

For pooled data from ELISA estimates, the effects of treatment compared to control were assessed by two way analysis of variance of square root transformed data, followed by

Dunnett's test. The number of replicate experiments is shown in the figure legends.

Results

IL-1α or TNFα induce secretion of IL-8, MCP-1 and RANTES from SMC

We have compared the differential ability of proinflammatory cytokines to induce chemokine production in human vascular SMC. IL-1α and TNFα over a range of concentrations (0.001–1000 ng ml^{-1}) induced IL-8, MCP-1 and RANTES production from cultured SMC. Figure 1 shows a representative (venous, explant) SMC culture from which chemokine secretion (ng ml^{-1}) has been measured after 24 h. The concentrations of IL-1α and TNFα that induced near maximum generation of each of these chemokines were 3 ng ml^{-1} and 30 ng ml^{-1}, respectively. These concentrations were used in all subsequent experiments.

In the experiments shown in Table 1, chemokine secretion from SMC derived from different venous and aortic tissues has been compared. When stimulated with IL-1α or TNFα, different cultures released different absolute amounts of chemokine protein. However, the trends in the responses of each culture, regardless of whether derived from vein or artery or explant or digest, were the same. In all cultures examined the production of IL-8 and RANTES by unstimulated SMC was less than could be detected by ELISA (<0.2 ng ml^{-1}). A small amount of MCP-1 was produced constitutively by SMC, the amount varied between the different cultures, but was in the range 0.3–4 ng ml^{-1}. IL-8 release induced by IL-1α was on average 3 fold that elicited by TNFα, within a range of 1.8–6 fold. In contrast, maximum levels of MCP-1 released by stimulation with IL-1α or TNFα were always similar. RANTES protein was not induced by 24 h treatment with IL-1α, whereas TNFα induced some RANTES production. Subsequent experiments revealed that larger quantities of RANTES protein were secreted at later time points in TNFα stimulated cells, for example 71±2.4 ng ml^{-1} (means±s.e.mean, $n=4$) RANTES were detected in the supernatants from venous derived SMC that had been treated for 72 h with TNFα, whereas the same cells produced only 2.9±0.15 ng ml^{-1} (mean±s.e.mean, $n=4$) RANTES when stimulated with IL-1α for 72 h.

IL-1α and TNFα upregulate chemokine mRNA expression in human SMC

Northern blot analysis indicated that IL-1α and TNFα both induced chemokine secretion via the upregulation of mRNA expression. IL-1α induced both IL-8 and MCP-1 mRNA with peak levels of expression at 4–6 h post-stimulation. MCP-1 mRNA expression was prolonged compared to IL-8 mRNA which started to decrease after 24 h stimulation. The small amount of RANTES mRNA induced in response to IL-1α peaked at 24 h post-stimulation (Figure 2a). As shown in Figure 2b, RANTES mRNA expression stimulated by TNFα occurred much later than either IL-8 or MCP-1 with a peak expression at 48 h. IL-8 and MCP-1 mRNA were both induced early by TNFα and again MCP-1 mRNA expression was upregulated for longer than IL-8 mRNA.

Effect of IL-10 and IL-13 on SMC chemokine secretion

The T-cell cytokines IL-10 and IL-13 are known to inhibit chemokine production from monocytes. We found that IL-13 or IL-10 alone did not significantly alter basal IL-8 or MCP-1 secretion from cultured SMC (not shown). The addition of IL-13 to SMC stimulated with either IL-1α (3 ng ml^{-1}) or TNFα (30 ng ml^{-1}) caused a significant concentration-dependent increase in both IL-8 and MCP-1 secretion (Figure 3). The highest concentration of IL-13 used (10 ng ml^{-1}) induced an 84±35% increase in IL-8 and a 111±20% increase in MCP-1 production stimulated by TNFα. These chemokine levels were significantly ($P<0.01$) enhanced above control levels. IL-13 also significantly increased IL-1α induced IL-8 and MCP-1, 26±13% and 92±22%, respectively, above control values. The smaller effect of IL-13 when IL-1α was used as the stimulus did not appear to be the result of a saturation of the cells ability to produce IL-8, since similar results were obtained with a submaximum concentration (0.3 ng ml^{-1}) of IL-1α as the stimulus (not shown). The results obtained were specific to

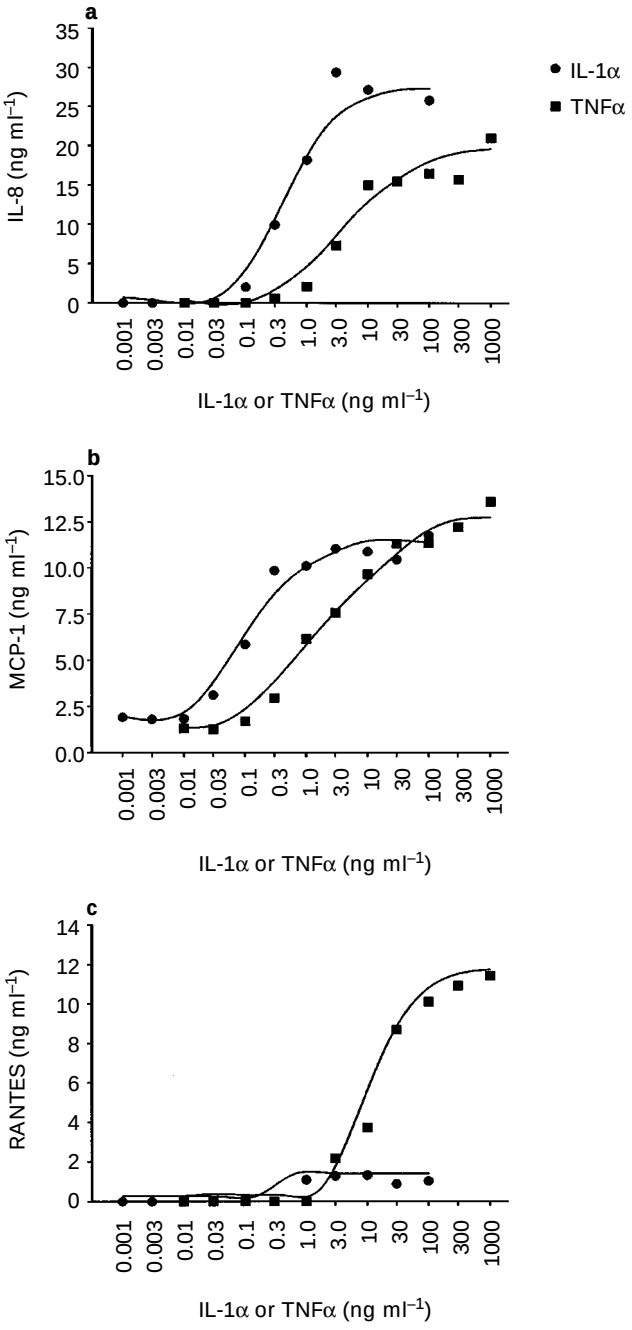

Figure 1 Chemokine secretion by human vascular SMC stimulated with IL-1α or TNFα. SMC were grown to confluence in 24 well plates and serum starved for 24 h. IL-1α or TNFα were added in 0.5 ml serum free DMEM at the concentrations shown. After 24 h the supernatants were removed and assayed by ELISA for (a) IL-8, (b) MCP-1 and (c) RANTES protein. Results are from a representative culture derived from venous tissue. Similar results were obtained with cells derived from 2 further venous tissues, each from a different donor. The results are mean chemokine levels (ng ml^{-1}) obtained from duplicate culture wells. Pooled standard deviation indicating variations of replicates in each assay was 3.0, 0.75 and 0.38 for the IL-8, MCP-1 and RANTES assays, respectively.

IL-13 and could not be reproduced by another T-cell cytokine, IL-10, which had no effect. In 4 separate experiments neither IL-10 nor IL-13 affected TNFα induced RANTES secretion measured after 24 h stimulation (not shown). After 72 h treatment in serum-free medium in the presence of TNFα and IL-13, there was evidence of reduced SMC viability. Hence, it was not possible to determine whether IL-13 modulated RANTES production in the same way as IL-8 and MCP-1.

Table 1 Chemokine secretion by SMC stimulated for 24 h with IL-1α or TNFα

Tissue donor	*Tissue type*	*Treatment*								
		Untreated	*IL-1α*	*TNFα*	*Untreated*	*IL-1α*	*TNFα*	*Untreated*	*IL-1α*	*TNFα*
		IL-8 secretion (ng ml^{-1})			*MCP-1 secretion* (ng ml^{-1})			*RANTES secretion* (ng ml^{-1})		
I	Artery (digest) p5	<0.2	45	7.5	0.3	22	15	NT	NT	NT
I	Artery (digest) p6	<0.2	51	17	4.2	7.2	7.5	<0.2	0	3.7
II	Vein (digest) p5	<0.2	50	19	0.6	15	15	<0.2	0.5	15
II	Vein (digest) p6	<0.2	48	26	2.6	25	29	<0.2	0.7	4.6
III	Vein (explant) p4	<0.2	39	13	0.6	27	25	<0.2	0	15
IV	Vein (explant) p5	<0.2	110	31	0.8	15	13	NT	NT	NT
	Mean ± s.e.mean	<0.2	57 ± 11	19 ± 3	1.5 ± 0.7	18.5 ± 3	17 ± 3	<0.2	0.3 ± 0.26	9.5 ± 3

SMC were grown to confluence in 24 well plates and serum starved for 24 h. The cells were treated with IL-1α (3 ng ml^{-1}) or TNFα (30 ng ml^{-1}) for 24 h. Supernatants were removed and assayed for IL-8, MCP-1 and RANTES by ELISA. The results are mean amount of chemokine (ng ml^{-1}) determined from 2 culture well supernatants. The bottom row shows mean ± s.e.mean for the pooled data. NT = not tested. The cells used in these experiments were between passage 4 and 6 (p4 – p6).

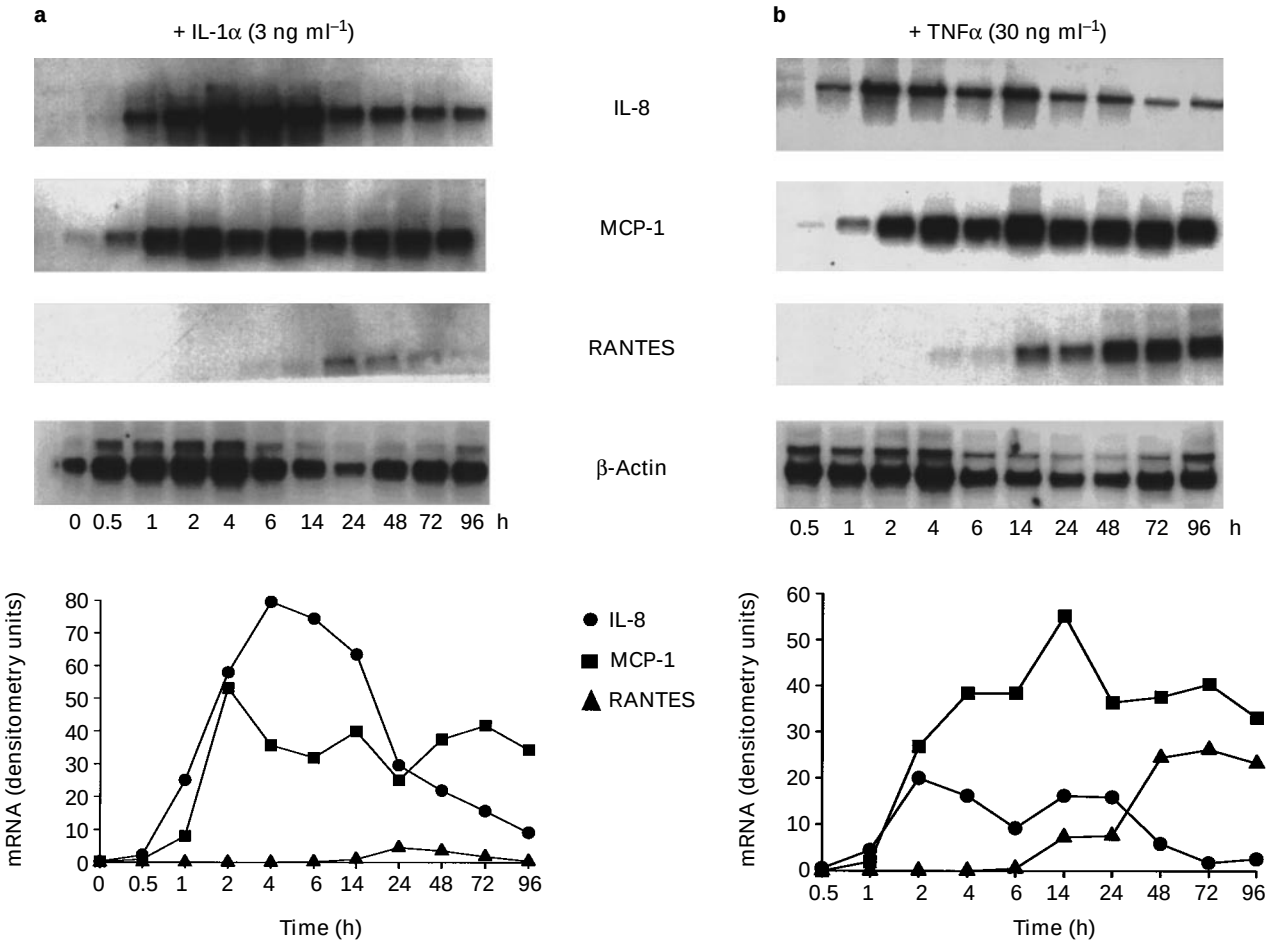

Figure 2 Expression of chemokine mRNA by human SMC stimulated with (a) IL-1α or (b) TNFα. SMC were grown to confluence in 25 cm^2 tissue culture flasks. Following 24 h serum starvation, the cells were stimulated with IL-1α (3 ng ml^{-1}) (a) or TNFα (30 ng ml^{-1}) (b) for the times indicated between 0.5 and 96 h. The extracted RNA was analysed by Northern blotting as described in Methods. Relative amounts of IL-8, MCP-1 and RANTES mRNA were measured by densitometry. The values shown in the graph have been corrected for differences in loading according to the *β*-actin control. Data shown are from a representative experiment with aortic tissue SMC initially isolated using the digestion method. Similar responses were seen in SMC derived from 2 other venous explant cultures.

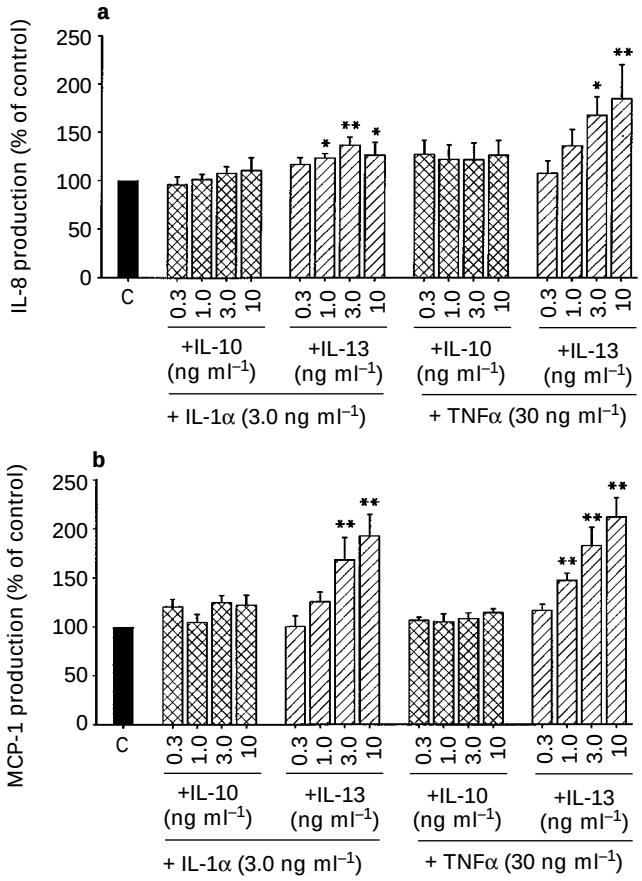

Figure 3 Effect of IL-10 and IL-13 on IL-1α and TNFα induced chemokine production in SMC. SMC were grown in 24 well plates and serum starved for 24 h. The cells were then pretreated for 1 h with IL-13 or IL-10 at the indicated concentrations (0.3–10 ng ml^{-1}). IL-1α (3 ng ml^{-1}) or TNFα (30 ng ml^{-1}) were added still in the presence of IL-10 or IL-13, and the incubation was continued for 24 h. The supernatants were removed and assayed for (a) IL-8 and (b) MCP-1. The results have been expressed as a percentage of the IL-1α or TNFα only control (c), which is given as 100%. Results are expressed as mean±s.e.mean for $n=5$ or 6 independent experiments (using the tissue samples detailed in Table 1). Significance was assessed by Dunnett's test, $^{**}P<0.01$, $^{*}P<0.05$.

Effect of IL-13 on IL-8 and MCP-1 mRNA expression

Changes in IL-13-stimulated chemokine release appeared to be accounted for by increased mRNA expression as shown in Figure 4. IL-13 was able to upregulate both IL-8 and MCP-1 mRNA induced by TNFα (Figure 4a) or IL-1α (Figure 4b), this was particularly evident after 6 h stimulation. The results shown are from a single venous (explant) culture. This culture was representative of 4 of the (venous explant) cultures examined, although in one further culture from different donor TNFα induced IL-8 mRNA was consistently (in 3 independent experiments) inhibited by 10 ng ml^{-1} IL-13, while MCP-1 mRNA was increased. Since this was also a venous culture which had been derived initially by an explant method, we are unable to explain this discrepancy.

Discussion

These results suggest that the production both spatially and temporally of the chemokines IL-8, MCP-1 and RANTES by human vascular SMC is differentially induced by IL-1α and TNFα and that this induction can be upregulated by IL-13 but not by IL-10. Furthermore, the data suggest that the vascular SMC could play a key role in the development of the atherosclerotic plaque, not only in its well characterized production of initimal thickening, but as both a source of chemokines which will attract and activate additional monocytes and T-cells and as a cell responsive to the T-cell derived IL-13. Indeed the vascular SMC can no longer be regarded as a structural cell but as an active player in the interaction between vasculature and the immune system.

The 'response to injury hypothesis' (Ross, 1993), proposes that atherosclerotic lesions develop at regions of endothelial dysfunction. At these sites stimulating factors including PDGF from activated platelets and circulating LDL are believed to induce the expression of adhesion molecules, such as the vascular cell adhesion molecule-1 (VCAM-1; Khan *et al*., 1995) and chemoattractants like MCP-1 (Taubman *et al*., 1992; Cushing *et al*., 1990) by the endothelium and underlying smooth muscle cells. These essential signals cause circulating monocytes and T-lymphocytes to attach to and migrate between endothelial cells. We have shown here that in cultured SMC, IL-1α and TNFα are potent stimuli for MCP-1 expression, in agreement with previously published findings (Wang *et al*., 1991; Seino *et al*., 1995). We also found that IL-1α and TNFα were able to stimulate production of much greater amounts of MCP-1 mRNA and protein, than were induced by PDGF or minimally oxidized LDL (data not shown). Hence, although LDL and PDGF may be important stimuli for the initiation of atherosclerotic lesions, we propose that IL-1α and TNFα present in atherosclerotic lesions may additionally amplify inflammatory cell infiltration via upregulation of SMC chemokines.

In addition to MCP-1 we have also shown that IL-1α or TNFα stimulated SMC produce the neutrophil and T-cell chemoattractant, IL-8. Since neutrophils are not a predominant cell type found in atherosclerotic plaques, the role of IL-8 may be related to its ability to recruit lymphocytes.

RANTES is a chemokine which attracts monocytes and T-cells, two cell types which account for over 20% of coronary plaque cells. The predominant T-cell in atherosclerotic lesions is CD4+ (Miller *et al*., 1995), which is also the major target of RANTES (Schall *et al*., 1990). This implies that RANTES may be an important chemokine in the development of lesions. We have shown here that TNFα is a more potent stimulus than IL-1α for RANTES production in vascular SMC. RANTES production has previously been described in human aortic SMC (Kauser *et al*., 1996) and airway SMC (John *et al*., 1997) and we and others have previously shown it to be produced by the smooth muscle-related kidney mesangial cell (Pattison *et al*., 1994; Robson *et al*., 1995) by human synovial fibroblasts (Rathanaswami *et al*., 1993; Jordan *et al*., 1996) and by endothelial cells (Marfaing-Koka *et al*., 1995; Kauser *et al*., 1996). In mesengial cells, synovial fibroblasts and SMC there is also a marked differential regulation of RANTES expression in response to IL-1α and TNFα, with TNFα being the superior stimulus. We found that the very poor induction of RANTES by IL-1α was much more marked in vascular SMC. In endothelial cells TNFα or IL-1α alone induced little or no RANTES while the combination of TNFα and IFNγ were required to stimulate strong RANTES production (Marfaing-Koka *et al*., 1995). RANTES is also different in that it is induced much later than IL-8 or MCP-1, it has previously been suggested that this is due to the requirement of an intermediary protein as a preliminary to RANTES induction (Rathanaswami *et al*., 1993). The kinetics of TNFα induced RANTES expression suggest that this chemokine may be involved in the recruitment and activation of T-cells over long periods.

IL-10 and IL-13 are produced in humans by activated CD4+ and CD8+ T-cells with Th0, Th1 and Th2 characteristics (Yssel *et al*., 1992; Zurawski & De Vries, 1994). They are also produced by mast cells (Moore *et al*., 1993; Burd *et al*., 1995), B-cells (Burdin *et al*., 1993; Fior *et al*., 1994) and some non-haematopoietic cells including keratinocytes (Michel *et al*., 1994; Enk *et al*., 1995). The presence of activated T-cells, together with monocytes and macrophages in atherosclerotic

lesions suggests that both IL-10 and IL-13 may be present. Simultaneous stimulation of monocytes with LPS and IL-13 or IL-10 down regulates the generation of the following LPS-induced chemokines, cytokines and haematopoietic growth factors: IL-1α/β, TNFα, IL-6, IL-8, GMCSF (De Waal Malefyt, 1991; 1993). IL-13 and IL-10 may also inhibit inflam-

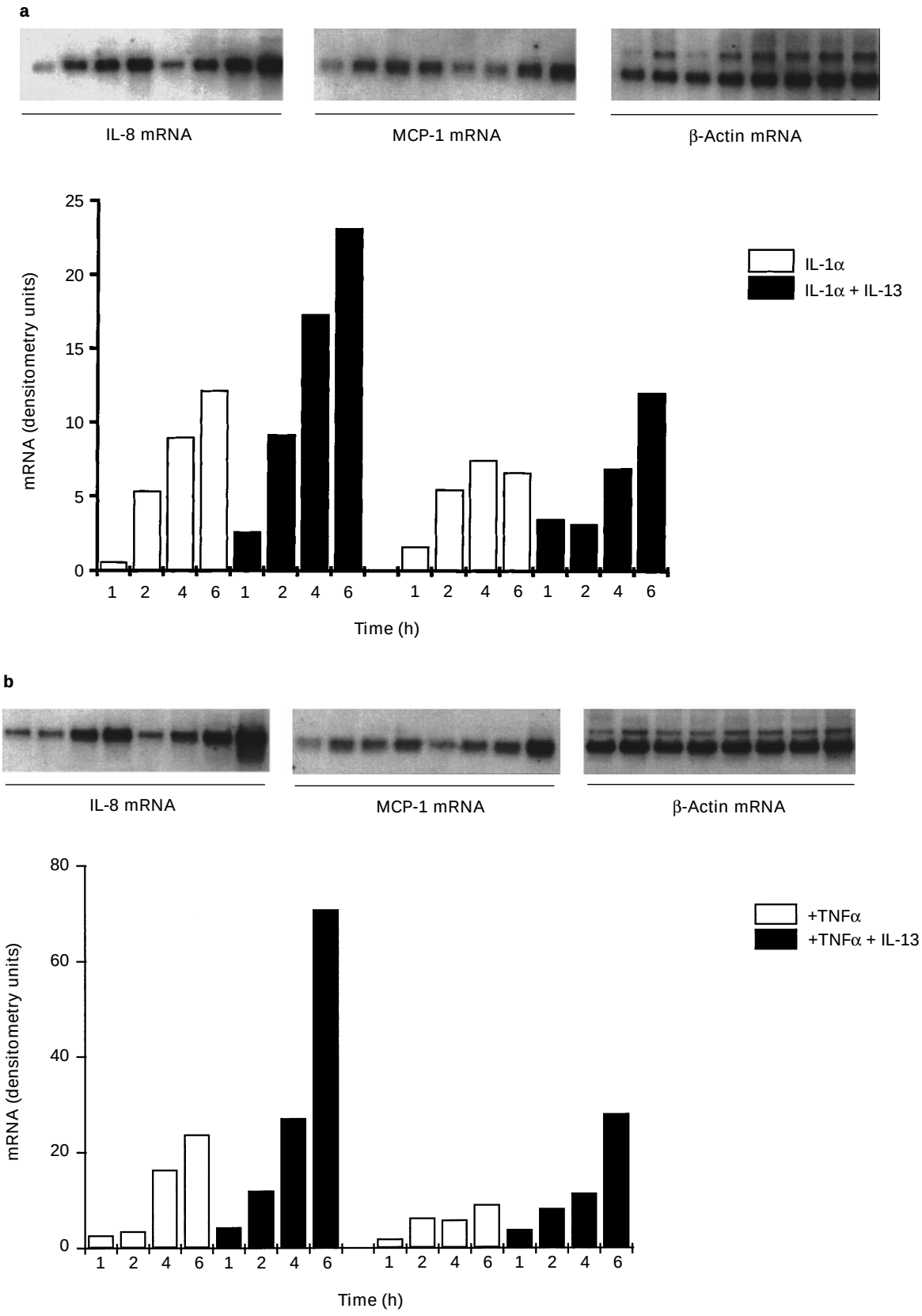

Figure 4 Effect of IL-13 on IL-8 and MCP-1 expression induced by IL-1α or TNFα. Cells were grown in 25 cm^2 flasks or 6 well plates and depleted of serum for 48 h. The cells were left untreated or pretreated for 1 h with 10 ng ml^{-1} IL-13 then IL-1α (a) or TNFα (b) was added for 1, 2, 4 or 6 h as shown. RNA was extracted and analysed by Northern blotting. Relative amounts of IL-8 and MCP-1 mRNA were measured by densitometry. The values shown in the graph have been corrected where necessary for differences in loading according to the β-actin control. Data shown are from a representative experiment, similar results were seen in 2 other venous cultures, each from a different donor.

matory responses by upregulation of the IL-1α receptor antagonist (De Waal Malefyt *et al*., 1993; Moore *et al*., 1993).

However, the modulatory role of IL-13 is complex and its effects appear to be stimulus, time and target cell specific. For example, while IL-13 added at the beginning or a few hours before stimulation of monocytes or macrophages with LPS, inhibits the production of cytokines, pretreatment for 20 h or more primes the cells for increased production of IL-12 and TNF (D'Andrea *et al*., 1995). IL-13 can also activate neutrophils and upregulate their production of IL-8 (Girard *et al*., 1996). Our results confirm those suggested by a number of other studies, that the 'anti-inflammatory' effects of IL-13 may be largely restricted to short pretreatment (1 h) of monocytes and that many of the effects of IL-13 on non-haematopoietic cells may be proinflammatory. We have shown that IL-13 enhances IL-1α or TNFα induced IL-8 and MCP-1 by up to 2 fold in SMC. Previous studies from our laboratory have shown that IL-13 upregulates IL-1α-induced IL-8 in a colonic epithelial cell line HT-29 (Kolios *et al*., 1996). IL-13 has also been found to amplify IL-6 production in keratinocytes, endothelial and mesothelial cells (Sironi *et al*., 1994; Derocq *et al*., 1994). Expression of VCAM-1 in endothelial cells is also enhanced by IL-13 (Bochner *et al*., 1995). Upregulation of VCAM-1 which promotes the endothelial adhesion of T-cells and monocytes in coronary atherosclerotic plaques (O'Brien *et al*., 1993) may reflect the participation of T-cell derived IL-13.

The inability of IL-10 to affect responses of target cells that respond to IL-13 is not without precedence, since this cytokine did not induce or amplify VCAM-1 on endothelial cells that responded to IL-13 (Sironi *et al*., 1994). The action of IL-10 may also be stimulus-dependent, since it can inhibit the production of IL-1α in astrocytes and microglia stimulated with components of gram-positive bacteria, but has no effect when IL-1α or TNFα are used as stimuli (Frei *et al*., 1994).

We have shown here for the first time that vascular SMC can respond to IL-13 and hence suggest the presence of a functional IL-13 receptor. In our experiments IL-13 alone did not induce significant amounts of either IL-8 or MCP-1, but it did upregulate the production of chemokines in response to IL-1α or TNFα. The mechanism of action appeared to be by upregulation of chemokine mRNA expression. We did not find any effect of either IL-10 or IL-13 on the secretion of RANTES by SMC. Human airway epithelial cells can also be stimulated to release RANTES which is similarly unaffected by IL-10 or IL-13 (Berkman *et al*., 1996). These differences may be due to the requirement of an intermediate protein for RANTES expression (Rathanaswami *et al*., 1993), which is not induced by IL-13. RANTES induced by a combination of TNFα and IFNγ in endothelial cells was partially inhibited by IL-13 (Marfaing-Koka *et al*., 1995), as was RANTES production by human airway smooth muscle cells (John *et al*., 1997), the difference here may be due to the inclusion of IFNγ as an additional stimulus.

Numerous cell types are present in atherosclerotic plaques, this combined with the pleiotropic effects of IL-13 indicates that the overall immunomodulatory effects of this cytokine are complex. Although IL-13 may inhibit proinflammatory cytokine and chemokine production by monocytes, it can clearly upregulate chemokine production by the smooth muscle cells. Our data suggest that IL-13 may contribute to the infiltration of monocytes and T-cells into the vessel wall via the upregulation of the chemokines IL-8 and MCP-1.

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