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Regulated chemokine gene expression in mouse mesothelioma and mesothelial cells: TNF-α upregulates both CC and CXC chemokine genes

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Abstract. Many cancers express an array of chemokines which have the capacity to modulate the nature and function of intratumoural leukocyte infiltrates. In malignant mesothelioma (MM) neither the chemokine signalling networks nor their regulation have been investigated despite the prominence of leucocytic infiltrates in both clinical and experimental tumours. In this study, we examined constitutive and cytokine-regulated expression of CC and CXC chemokine genes in mesothelioma and mesothelial cell cultures derived from two different mouse strains (BALB/C and CBA/CaH). In mouse MM and mesothelial cells MCP-1/JE, GRO-α/KC and RANTES were expressed whereas MIP-1α and MIP-2 were infrequently expressed. Comparison of basal chemokine expression showed that GRO-α/KC mRNA was overexpressed in the malignant cells whereas MCP-1 gene expression and release was down-regulated. Treatment of mesothelioma cells with IL-4, IFN-γ or TNF-α revealed that chemokine genes could be more responsive to cytokines in the malignant compared to their mesothelial cells. TNF-α was consistently the most potent positive regulator of both CC and CXC chemokine expression and MCP-1 release. The present study for the first time provides a mechanistic insight into the differential regulation of chemokine expression in malignant mesothelioma cells and has implications for mesothelial chemokine signalling in mouse models.

Introduction

Chemokines are a large family of chemotractant cytokines involved in the regulation of cell trafficking which are believed to play a significant role in influencing tumour progression (1-3). Chemokine signalling influences the recruitment and movement of leukocytes and other cell types and can also provide proliferative, survival and angiogenic stimuli (4,5). It is well recognised that normal cell types play an essential role in tumour progression and that chemokines and other soluble factors derived from tumour cells are involved in recruitment of these cells and modulation of their activity. In this manner chemokines produced by tumour cells may play a role directly or indirectly in tumour neovascularisation and could also modify anti-tumour immune response. The effect of malignant transformation upon the regulation of chemokine expression is of interest because of these implications for biological therapies which target tumour vasculature and for immunotherapies.

A major focus of our laboratory has been the investigation of immunological therapies to induce immunity to malignant mesothelioma (MM) in syngeneic mouse models of the disease. Mesothelioma is a particularly aggressive malignancy of the serosal surfaces which is most frequently associated with exposure to asbestos (reviewed in ref. 6). The cell lines used in these studies provide one of the few animal models of cancer in which the original tumours have been induced by the agent which is believed to be the causative agent of the human tumour (i.e. asbestos), and have been extensively characterised both in this laboratory (7-9) and by others (10,11). A feature of these murine tumours is a prominent inflammatory infiltrate composed predominantly of monocytes which may comprise upward of 50% of the cells (10). A number of studies have shown that mesothelial cells can regulate leukocyte trafficking during inflammation through chemokine production (12-15) and various researchers have demonstrated *in vitro* that inflammatory cytokines can regulate chemokine expression in mesothelial cells (15-17). However, although IL-8 has been implicated in the progression of MM in experimental models (18,19) the function and regulation of chemokine networks has not been characterised in mesothelial derived malignancy.

The aim of the present study was to determine the capacity of mesothelioma cells from different mouse strains to express and regulate chemokine genes in response to inflammatory mediators. Primary mouse mesothelial cultures from these mouse strains were used as a basis for comparison and studies using these also provided useful insights into both mesothelial cell biology and strain specific chemokine responses.

The CCL chemokines MCP-1/JE (CCL2) and RANTES (CCL5) have been implicated in the recruitment of tumour

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associated macrophages in both mouse tumour models and human disease (2,20). The murine angiogenic CXC chemokines, GRO-α/KC and MIP-2 are homologues of the human GRO chemokines and are believed to serve similar functions in the mouse to IL-8 (CXCL8) in humans (21). Since chemokine production by tumour cells is likely to be relevant to tumour progression both by influencing the intratumoural microenvironment and also as a response to immunotherapy the effects of both Type I (IFN-γ and TNF-α) and Type II (IL-4) cytokines, each of which may be elaborated by immune effector cells or has been used in immunotherapeutic approaches to cancer therapy, were examined. The availability of both malignant and normal primary cell cultures derived from two mouse strains, CBA/CaH and Balb/c, allowed investigation of differences in chemokine gene expression as a consequence of malignancy and genetic background.

Materials and methods

Mesothelioma cell culture and reagents. The murine MM cell lines AB1, AB12, AC29, AC34 and AE17 were used in this study. These cell lines were originally derived by intraperitoneal inoculation of crocidolite asbestos in BALB/C mice (AB1 and AB12), CBA/CaH mice (AC29 and AC34) or C57BL/J mice (AE17) as described previously (7,22). All cells were cultured and maintained in medium R5, which is RPMI-1640 plus 5% heat-inactivated foetal bovine serum (FBS) (Invitrogen, Victoria, Australia), 300 mM L-glutamine (Invitrogen), 120 μg/ml penicillin (Invitrogen) and 100 μg/ml gentamicin (Invitrogen). All cell cultures were grown at 37˚C in a 5% CO_2 humidified atmosphere.

Mesothelial cell culture. Normal mesothelial cells were isolated from the anterior peritoneal wall of 8-10-week-old female Balb/c or CBA/CaH mice (Animal Resource Centre, Murdoch, WA, Australia) essentially as described by Foley-Comer *et al* (23). Briefly, peritoneal tissue from 3 mice was incubated in 0.25% trypsin and 0.02% EDTA in DMEM medium (Trace Scientific, Victoria, Australia) at 37°C for 30 min with gentle agitation. The tissue was removed and cells collected by centrifugation at 1000 rpm (200 g) for 5 min at room temperature. Cells were resuspended and transferred to culture flasks in mesothelial culture medium, consisting of DMEM plus 15% FBS (Invitrogen), 5 ng/ml epidermal growth factor (Roche Diagnostics, N.S.W., Australia), 0.4 μg/ml hydrocortisone, 4 mM L-glutamine (Invitrogen), 120 penicillin 100 U/ml (Invitrogen) and 50 μg/ml streptomycin (Invitrogen). All cell cultures were grown at 37˚C in a 5% CO_2 humidified atmosphere. Peritoneal mesothelial cells (PMC) demonstrated a characteristic cobblestone morphology in culture and were used at passages 2-3.

Chemokine expression and release experiments. To determine the concentration-dependent effect of cytokines upon MM and PMC, chemokine expression and release, $5x10^5$ cells/well were seeded in 6-well plates and cultured for 24 h. The cultures were then incubated for 24 h with various concentrations of one of three cytokines: IFN-γ (Sigma-Aldrich, N.S.W., Australia, 1-100 ng/ml), TNF-α (Sigma, 1-100 ng/ml) or IL-4 (Sigma, 1-100 ng/ml). Total cellular RNA was extracted from cells as described below. Culture supernatants were harvested, clarified by centrifugation and stored at -80˚C for ELISA. All experiments were conducted in triplicate.

Table I. Oligonucleotide primers.

Gene	Primer sequence 5'-3'	GenBank acc. no.
MCP-1/JE	CAGCACCAGCCAACTCTCACT AAGGCATCACAGTTCGAGTCA	NM_011333
GRO-α/KC	CACCATGATCCCAGCCACCCG TTACTTGGGGACACCTTTTAG	NM_008176
RANTES	CCCTCACCATCATCCTCACT CCTTCGAGTGACAAACACGA	NM_013653
MIP-2	CACTTCAGCCTAGCGCCAT GTCAGTTAGCCTTGCCTTTG	NM_009140
MIP-1α	CCTCTGTCACCTGCTCAACA GATGAATTGGCGTGGAATCT	NM_011337
CSF1	GACCCTCGAGTCAACAGAGC TGTCAGTCTCTGCCTGGATG	NM_007778
CSF2	TGGTCTACAGCCTCTCAGCA CCGTAGACCCTGCTCGAATA	NM_009969
CCR2	GGGTCATGATCCCTATGTGG TCCATGAGCAGTGGTTTGAA	NM_009915
CXCR2	CATCAGCATGGACCGCTAC GCAGGGCCAGAATTACTGAT	NM_009909

Reverse transcription PCR (RT-PCR) and real-time RT-PCR. Total RNA was prepared from cultures of cell lines using Ultraspec reagent (Biotecx, TX, USA) according to the manufacturer's instructions, resuspended in 1 mM sodium citrate and stored at -80˚C. Prior to RT-PCR, contaminating DNA was removed from the RNA using RQ1 DNAse (Promega, N.S.W., Australia). First strand cDNA synthesis was carried out using AMV reverse transcriptase (Reverse Transcription System, Promega) and 1 μg total RNA primed with random hexamers in a final volume of 20 μl. Gene specific PCR primers were designed using the Primer 3 software (24) and sequences are shown in Table I. Conventional PCR was performed in a 25 μl reaction comprising 2 mM $MgCl_2$, DNA polymerase buffer (Fisher-Biotec, W.A., Australia), 200 μM dNTP, 100 nM each primer, 1U Taq DNA polymerase (Fisher-Biotec), and 2 μl of cDNA. Amplification reactions were run in PTC-100 (MJ Research, MA, USA) cyclers and amplified products analysed by agarose gel electrophoresis, then photographed using a Kodak EDAS 120 digital camera system.

Real-time PCR was performed using a RotorGene 2000 real-time amplification instrument (Corbett Research, N.S.W., Australia) with Sybr Green I detection chemistry. Reactions were performed in a 20 μl volume with 2 μl cDNA, 2-4 mM $MgCl_2$, 200 μM dNTP, 1 U Taq DNA polymerase (Fisher-Biotec), 0.1-0.5 μM of each primer, $5x10^{-5}$ SYBR Green I

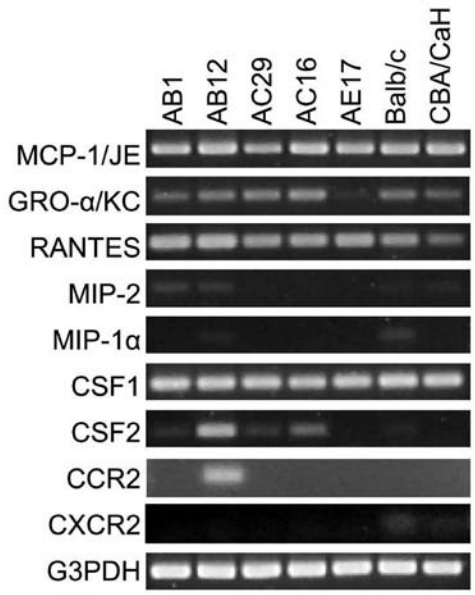

Figure 1. Chemokine and chemokine receptor mRNA expression in mouse mesothelioma and mesothelial cells. Total RNA isolated from cells was analysed by 2-step RT-PCR using gene-specific primers.

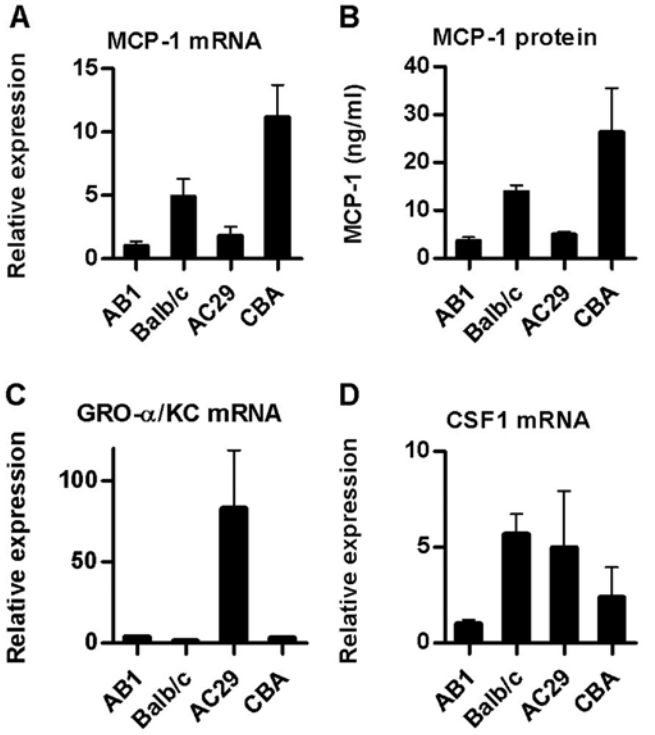

Figure 2. Basal gene expression (A) MCP-1/JE (C) GRO-α/KC (D) CSF1 and protein release (B) MCP-1/JE in mouse mesothelioma and mesothelial cells. Total RNA isolated from cells was analysed by 2-step real-time RT-PCR using gene specific primers. Basal gene expression is expressed as mRNA levels relative to the lowest expressing cultures following normalisation by reference gene expression. MCP-1 concentration was measured by sandwich ELISA assay. Results are means and SD for three independent experiments.

(Invitrogen), DNA polymerase buffer (Fisher-Biotec). A typical protocol comprised 95˚C for 5 min followed by 45 cycles of 95˚C for 20 sec, 55-60˚C for 20 sec, 72˚C for 45 sec and 80-87˚C for 15 sec, then an additional 60 sec at 72˚C. Fluorescence data were acquired at 72˚C and 80-87˚C (optimised for each assay). Primer and $MgCl_2$ concentration as well as annealing temperature were optimised for each assay. To confirm amplification specificity a melt curve analysis was performed at the end of each run.

Standard curves were generated using serially diluted cDNA. Real-time PCR assays were conducted in duplicate for each sample and control. The threshold cycle (CT) was determined automatically by the RotorGene software (v4.3) using the dynamic tube normalisation setting. In order to allow for sample-to-sample variability, gene expression data were normalised to levels of expression of reference (housekeeping) genes. There were four reference gene assays for which the primers were: hypoxanthine phosphoribosyl-transferase 1 (HPRT1): forward (5'-tgacactggtaaaacaatgca-3'), reverse (5'-ggtccttttcaccagcaagct-3'); glyceraldehyde-3-phosphate dehydrogenase (G3PDH): forward (5'-accacagtccatgccatcac-3'), reverse (5'-tccaccaccctgttgctgta-3'); ubiquitin C (UBC): forward (5'-aggtcaaacaggaagacagacgta-3'), mouse reverse (5'-tcacacccaagaacaagcaca-3'); 18S ribosomal RNA (18S): forward (5'-gtaacc cgttgaaccccatt-3'), reverse (5'-ccatccaatc gtagtagcg-3'). Primer sequences for the UBC and HPRT1 genes were obtained from the RTPrimerDB at medgen.ugent.be/rtprimerdb (25). Real-time assays were performed on the relevant samples and the most stable reference genes were determined using the geNorm software (v3.3) and used to generate a normalisation factor for each sample essentially as described by Vandesompele *et al* (26). Relative expression of the target gene was normalised using this factor and expressed as mean ± standard deviation relative to a control or calibrator sample.

ELISA. MCP-1 levels in culture supernatants were quantitated by sandwich ELISA assay. An OptEIA mouse MCP-1 ELISA set (BD Biosciences, N.S.W., Australia) with a detection limit of 30 pg/ml MCP-1 was used according to the manufacturer's instructions.

Results

Expression of CXC and CC chemokine mRNA in mesothelioma cells. Unstimulated cultures of mouse mesothelioma cell lines and PMC were surveyed for the expression of specific chemokine mRNAs using conventional RT-PCR (Fig. 1). The mRNAs for MCP-1/JE, GRO-α/KC and RANTES were detectable in all mouse mesothelioma cell lines (except GRO-α/KC in AE17) and PMC whereas MIP-1α was very faintly detectable in Balb/c PMC and MIP-2 expression was absent (Fig. 1). We also examined expression of CSF1 and CSF2, two molecules which can function as monocyte chemoattractants (27); CSF1 was detected in all mouse MM and PMC cultures while CSF2 was detected in AB12 and weakly in AC16.

We also examined the relative basal expression of MCP-1/JE, GRO-α/KC and CSF1 by performing real-time RT-PCR upon RNA extracted from cultures of the AB1 and AC29 cell lines as well as PMC from both Balb/c and CBA/CaH mice. These are the two mouse MM models used most commonly in our laboratory and elsewhere (10,11). MCP-1/JE mRNA levels were higher in the mouse PMC relative to mesothelioma cultures (Fig. 2A). Assay of culture supernatants for MCP-1 protein by ELISA

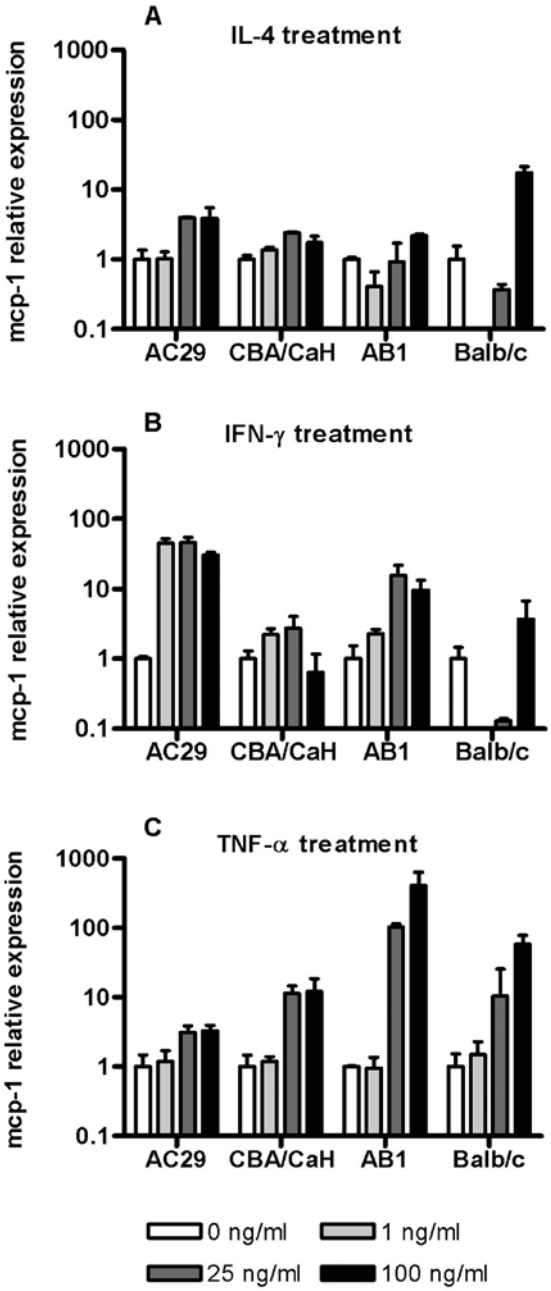

Figure 3. Induction of MCP-1/JE mRNA in murine mesothelioma and mesothelial cells in response to (A) IL-4, (B) IFN-γ or (C) TNF-α. Total RNA isolated from cells was analysed by 2-step real-time RT-PCR using gene specific primers. Values are expressed as mRNA levels relative to control cultures following normalisation by reference gene expression. Results are means and SD for three independent experiments.

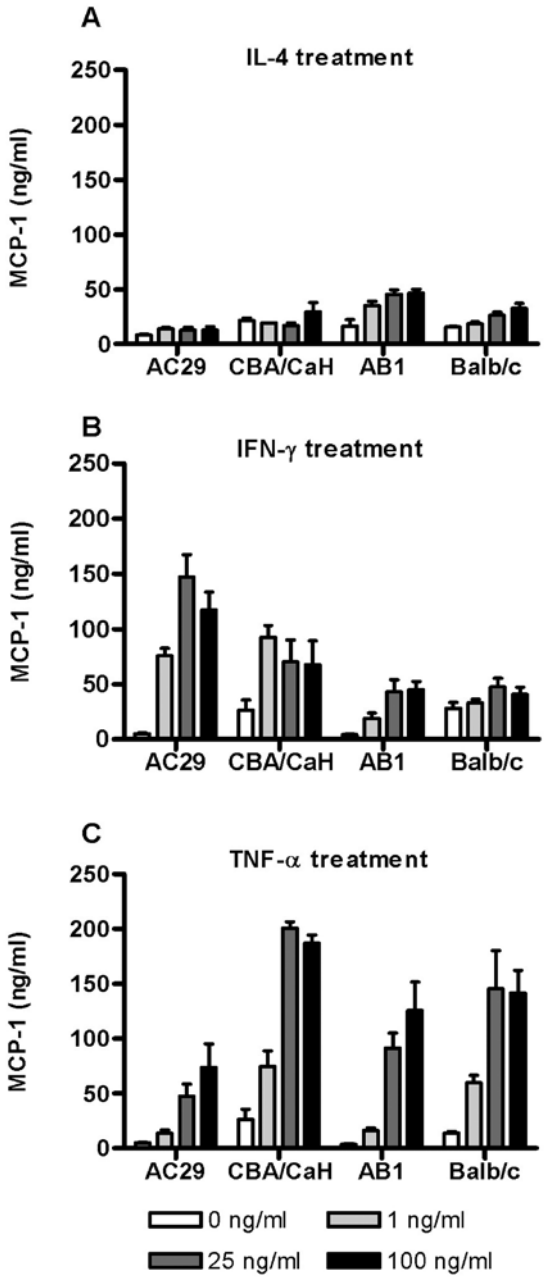

Figure 4. MCP-1/JE secretion by murine mesothelioma and mesothelial cells in response to (A) IL-4, (B) IFN-γ or (C) TNF-α. Supernatants were harvested at the same time point that cells were used for RNA isolation. MCP-1 concentration was measured by sandwich ELISA assay. Results are means and SD for three independent experiments.

confirmed these results (Fig. 2B). In contrast, expression of the CXC chemokine GRO-α/KC was higher in AC29 cells (26-fold) and to a lesser extent in AB1 (5-fold) than in normal PMC from the same strain (Fig. 2C).

Expression of the CCR2 and CXCR2 chemokine receptors. Since autocrine chemokine signalling has been described in some tumour types and mesothelial cell expression of CCR2 has been reported (28), we examined expression of CCR2 (receptor for MCP-1) and CXCR2 (receptor for GRO-α) mRNA in murine PMC and MM lines. CCR2 mRNA was expressed only in the AB12 murine mesothelioma cell line while CXCR2 mRNA was faintly detectable in PMC cultures but not in any of the tumour cell lines tested (Fig. 1).

Cytokine regulation of MCP-1 mRNA in mouse mesothelial and mesothelioma cells. To evaluate the potential for cytokines produced by tumour cells, stromal cells or inflammatory cells to regulate MCP-1 gene expression we determined the ability of IL-4, IFN-γ and TNF-α to control MCP-1 expression in mouse AB1 and AC29 MM cell lines as well as corresponding PMC cultures (Fig. 3). IL-4 induced a modest dose-dependent increase in MCP-1 mRNA in CBA derived cell but showed a biphasic response in Balb/c derived cells especially in PMC with

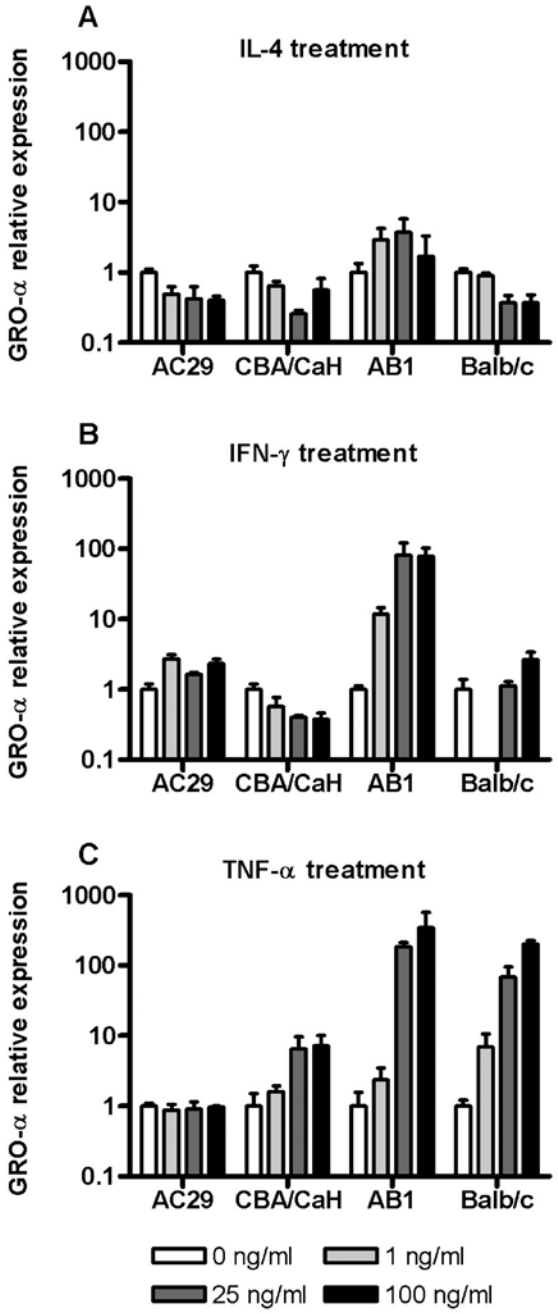

Figure 5. Induction of GRO-α/KC mRNA in murine mesothelioma and mesothelial cells in response to (A) IL-4, (B) IFN-γ or (C) TNF-α. Total RNA isolated from cells was analysed by 2-step real-time RT-PCR using gene-specific primers. Values are expressed as mRNA levels relative to control cultures following normalisation by reference gene expression. Results are means and SD for three independent experiments.

downregulation at low IL-4 concentrations to below the level of detection while high concentrations resulted in upregulation (Fig. 3A). A similar effect was seen when these cells (Balb/c PMC) were exposed to 1-25 ng/ml of IFN-γ (Fig. 3B). Overall the effects of IFN-γ upon MCP-1 mRNA were more profound in the tumour cell lines with dose-dependent upregulation especially in AC29 (45-fold) maximal at 1 ng/ml.

TNF-α consistently upregulated MCP-1 expression at concentrations ~25 ng/ml in all the cells, although the effects varied in magnitude (Fig. 3C). In CBA derived cells PMC were more responsive than tumour cells (AC29) while in contrast the largest upregulation was seen in AB1 cells (350-fold). These results indicated that mesothelioma cells retain and in some cases enhance the ability for cytokine regulated MCP-1 expression. They also reveal strain specific differences in both normal and tumour cell responses.

MCP-1 release by mouse mesothelial and mesothelioma cells. To further examine the dose-dependent effect of cytokine stimulation on MCP-1 expression, the levels of MCP-1 protein in the corresponding culture supernatants were assayed by ELISA (Fig. 4). In Balb/c derived cells (PMC and AB1) IL-4 stimulated a modest dose-dependent release of MCP-1 but had little effect in those derived from CBA/CaH (Fig. 4A). In contrast IFN-γ stimulated substantial upregulation of MCP-1 release by both AC29 and CBA/CaH cells even at 1 ng/ml with lesser effects in Balb/c cells (Fig. 4B). By comparison TNF-α induced MCP-1 release in all the cell types assayed (Fig. 4C). The PMC cultures were consistently more responsive than the corresponding tumour cell line although substantial upregulation also occurred. In PMC (Balb/c and CBA/CaH) maximum MCP-1 levels were induced by 25 ng/ml TNF-α (200 ng/ml and 145 ng/ml, respectively). The results indicated that on the whole MCP-1 release is consistent with changes in gene expression.

Cytokine regulation of GRO-α/KC and CSF1 mRNA in mouse mesothelial and mesothelioma cells. In addition to the studies of MCP-1 mRNA expression and protein release described above the mRNA expression of two other molecules which may impact upon tumourigenesis via chemotaxis or angiogenesis, GRO-α/KC and CSF1, were also determined in response to cytokine stimulation. On the whole IL-4 was inhibitory to GRO-α expression in all cells except AB1 (Fig. 5A). Similarly GRO-α mRNA was generally insensitive to IFN-γ except in AB1 cells where there was a large (80-fold) upregulation of this chemokine (Fig. 5B). Of note, in Balb/c PMC we observed a downregulation of GRO-α mRNA at 1 ng/ml IFN-γ which was not seen at higher concentrations.

As was found for MCP-1, TNF-α was the most consistent inducer of GRO-α gene expression with the exception of AC29 cells which were insensitive (Fig. 5C). GRO-α expression in Balb/c derived PMC and tumour cells (AB1) were most sensitive to TNF-α with 200- and 400-fold upregulation, respectively. The expression of CSF-1 mRNA was relatively insensitive to cytokine exposure in all cells, varying of the order of 2-3-fold across the dose range (data not shown).

Discussion

Many cancers express an array of chemokines and their receptors which may have a role in modulation of the leukocyte infiltrate. In MM this aspect of tumour biology has received limited investigation. Macrophages infiltrate many solid tumours including MM and in animal models of MM this infiltrate is an early feature of tumour development. This suggests that the tumour cells may be the source of these recruitment signals. Previous investigations of mesothelial cells have demonstrated that cytokines regulate expression and release of chemokines in these cells and may play an important

role in leukocyte recruitment and trafficking into body cavities (12,14-17). This prompted us to ask if mesothelioma cells retain this feature after transformation. To our knowledge the present study is the first to report on cytokine regulated expression of these molecules in MM cells. Furthermore, few studies have examined by comparison the effect of malignant transformation upon regulation of chemokine gene expression. We found that mesothelioma cells retain the capacity of their mesothelial counterparts for regulated expression of these molecules in response to cytokines. In some respects MM cells displayed a heightened response relative to PMC. As with other tumour types in which chemokine signalling has been much more extensively characterised this has implications for the nature of intercellular signalling within the mesothelioma microenvironment. In addition to chemokines we examined other factors which might contribute to monocyte recruitment to mesothelioma tumours and showed that CSF1 was universally expressed, although expression levels were not as responsive to cytokines. It was also of particular interest to be able to compare and contrast regulation of gene expression between mesothelial and mesothelioma cells derived from different experimental models.

The CC chemokines MCP-1 and RANTES were detected in all the mouse MM cell lines examined as well as mouse mesothelial cultures. Both MCP-1 and RANTES have protumourigenic activities and have been implicated in monocyte recruitment (3,20). A prominent macrophage infiltrate is a feature of a number of murine MM models including AC29 and AB1 (10). Quantitative analysis of MCP-1/JE basal expression levels in MM cells showed significantly higher mRNA and protein release in the corresponding mesothelial cultures. This confounds the notion that constitutive MCP-1 overexpression contributes to macrophage recruitment in these tumours. However, cytokine stimulation studies demonstrated that MCP-1 was significantly upregulated in response particularly to TNF-α. Previous *in vitro* studies have demonstrated expression of functional CCR2 receptors and chemotactic responses to MCP-1 (CCL2) in human mesothelial cells (28). More recently, Davidson *et al* (29) found infrequent chemokine receptor expression in MM and reactive mesothelium and the absence of CCR2 although CXCR2 was not studied. We did not find expression or induction of CCR2 mRNA in either murine MM or PMC which was consistent with this latter study of their reported human counterparts.

CXC chemokines as a family display a range of functions including leukocyte chemotaxis and angiogenesis, each of which has been implicated in tumour progression. IL-8 is the only chemokine which has been previously implicated in MM progression through experimental data (18,19) with evidence of autocrine growth signalling *in vitro* and of *in vivo* tumour growth promotion although this avenue of research does not appear to have been pursued. The complexity and interspecies diversity of the chemokine system does not allow for a direct correlation to be drawn between the roles for human and murine chemokines. There is no direct mouse homologue for IL-8/CXCL8, however, it has been demonstrated that the murine chemokines KC/GRO-α and MIP-2 are functionally analogous (21). Notably, we found almost a 30-fold overexpression of KC/GRO-α mRNA in the mouse AC29 cell line relative to mesothelial cultures as well as insensitivity to TNF-α. Such constitutive overexpression of KC/GRO-α is a feature which has been implicated in the tumourigenicity of other experimental tumours (30,31). The absence of expression of the receptor CXCR2, even after cytokine stimulation, suggests that autocrine signalling by KC/GRO-α in murine MM cells, at least via this pathway, may not be prominent in these tumour cells.

In cytokine stimulation studies TNF-α was consistently the most potent upregulator of chemokine expression and MCP-1 release with the exception of the mouse AC29 cells. This has implications for *in vivo* chemokine production and is consistent with some current hypotheses regarding the role of tumour cells in shaping their microenvironment (32,33). We have shown that *in vitro*, MM cells are quite resistant to the cytotoxic effects of TNF-α (34) and in murine tumours TNF-α levels are quite high (10). Previously in one murine MM tumour line we observed growth promotion by TNF-α even at 100 ng/ml (34). Both MCP-1 and RANTES have been shown to induce TNF-α production in macrophages (20). There is ample evidence supporting involvement of tumour cell derived chemokines and other factors in macrophage recruitment and gene expression. The induction of TNF-α which may in turn participate in local signalling to enhance tumour cell chemokine production as reported here and elsewhere (32,33,35), is one mechanism by which tumour cell-macrophage crosstalk may enhance tumour growth.

Strain specific differences in the mouse immune response have been widely reported, however, few studies have investigated differential chemokine responses in mouse strains (36-38). Although there were differences in the basal gene expression levels in PMC cultures from CBA/CaH and Balb/c mice, differences in the response of PMC from the two strains to cytokine exposure were more evident (e.g. GRO-α in response to TNF-α, Fig. 5C). Our findings support previous suggestions that such strain specific differences need to be considered when interpreting and designing studies of mesothelial inflammation in mouse models.

Current evidence regarding the role of inflammatory mediators and leukocytes in cancer progression indicates that in many cancers there is a complex interaction with tumour cells which engenders an environment favourable to tumour growth. The data presented in this study demonstrate that mesothelioma tumour cells express a variety of chemokine genes and that these genes are regulated in response to cytokines known to be present in the tumour microenvironment. Importantly, this responsiveness may actually be enhanced in malignant cells. Of particular interest was TNF-α which upregulated both CC and CXC chemokines in MM cells and may participate in amplifying paracrine signalling loops. It may prove possible to target these pathways in MM and our characterisation here of murine models provides avenues for investigating these possibilities *in vivo*.

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