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Role of macrophage-colony-stimulating factor in regulating the accumulation and phenotype of tumor-associated macrophages

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Abstract In order to better define the role played by tumor-cell-derived macrophage-colony-stimulating factor (M-CSF) in regulating the recruitment and phenotype of tumor-associated macrophages, Polyoma large T-transformed fibroblastoid cell lines, derived from M-CSF-deficient osteopetrotic op/op mice and their phenotypically normal op/+ littermate controls, were inoculated into SCID (severe combined immunodeficiency) recipients and both the proportion and phenotype of the macrophages present within the tumors generated were determined. The results obtained indicate that, although tumors derived from M-CSF-deficient and M-CSF-producing tumor cell inoculate contain a similar proportion of macrophages, the macrophages isolated from tumors lacking M-CSF appear morphologically less mature and express lower levels of interleukin 1β, tumor necrosis factor α and FcRγII mRNA. Taken together, these data suggest that, although M-CSF does not appear to play a critical role in determining the macrophage content of these tumors, it does play a role in modulating the phenotype, and potentially the functional activity of the macrophages present within the tumor microenvironment.

Key words Tumor-associated macrophages · M-CSF · op/op mice

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Introduction

Most solid tumor masses, irrespective of histological type, contain a significant proportion of host-derived macrophages. Importantly, correlations have been noted between the presence and/or functional activity of such cells and various tumor parameters including local invasion, response to therapy, metastatic potential and prognosis [29, 31].

Tumor-associated macrophages are believed to be derived from both the differentiation of monocytes recruited into a tumor site from the peripheral blood [29, 31], and the localized proliferation of macrophage progenitor cells within the tumor microenvironment [15, 43]. Thus, factors that are chemotactic for monocytes and/or other more primitive macrophage progenitor cell types, or which promote the survival, proliferation and/or differentiation of these cells, might be expected to increase the total number of macrophages present within a tumor [31].

Macrophage-colony-stimulating factor (M-CSF) is an important regulator of macrophage production. It has been shown to function both as a monocyte-specific chemoattractant [48] and to promote the survival and differentiation of monocytes in vitro [45] and in vivo [9, 37] Significantly, M-CSF has also been shown to "activate" macrophages directly [36, 38, 41, 50, 51] and to "prime" these cells so that they can respond to other stimuli [49]. Phase 1 clinical trials exploring the anti-tumor activity of M-CSF are presently underway in a number of centers [2, 3].

In order to define further the role played by M-CSF in regulating the accumulation and phenotype of tumor-associated macrophages, transformed fibroblastoid cell lines derived from both M-CSF-deficient op/op mice and their phenotypically normal op/+ littermate controls were inoculated subcutaneously into SCID (severe combined immunodeficiency) mice and both the proportion and phenotype of the macrophages present within the tumors produced were determined. The data obtained suggest that, while tumor-cell-derived M-CSF does not appear to play a major

role in determining the total macrophage content, of at least these tumors, it does impact on both tumor growth and the phenotype of tumor-associated macrophages.

Materials and methods

Animals

(C57BL/6J×C3HeB/FeJ)F2 B6C3Fe (osteopetrotic) op/op mice, readily identifiable by virtue of their domed skulls and absence of teeth, and phenotypically normal (+/+ or op/+) littermate controls, were obtained from Jackson Laboratories (Bar Harbor, Me.). BALB/c-nu/nu mice, originally obtained from Harlan Sprague Dawley (Indianapolis, Ind.), BALB/c-SCID mice originally obtained from Dr. J. Dick (Hospital for Sick Children, Toronto, Ont.) and C3H/HeJ mice originally obtained from Jackson Laboratories, were bred and maintained in the Joint Animal Facility of the British Columbia Cancer Research Centre.

Cells lines

The simian-virus-40(SV40)-transformed simian fibroblastoid cell line COS7 [18] was obtained from the American Type Culture Collection (ATCC; Rockville, Md.).

op/op and op/+ fibroblastoid cell lines were derived respectively from the lungs of op/op mice and their phenotypically normal littermates. Briefly, lung tissue was pooled from two mice and finely minced using surgical blades. The resulting pieces were suspended in 10 ml RPMI medium (Stem Cell Technologies Inc., Vancouver, B.C.) containing 20% fetal calf serum (FCS; Hyclone) (RPMI/20%FCS), plated in a 10-cm tissue-culture dish (Falcon 3003; Becton Dickinson Labware, Mississauga, Ont.) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Outgrowth of fibroblastoid cells was evident after about 1 week, and the medium along with non-adherent cellular debris was removed and 10 ml fresh RPMI/20% FCS added. As the fibroblast layers became confluent, they were maintained by regular passage using 0.25% trypsin in citrate/saline (Stem Cell Technologies Inc.), and the FCS concentration in the medium was reduced to 10%.

The transformed fibroblastoid cell lines op/opPy and op/+Py were generated using an ecotropic retroviral vector (CTV-4Py) encoding the polyoma large T antigen (a generous gift from Dr. Robert Kay, Terry Fox Laboratory). Briefly, subconfluent monolayers of op/op and op/+ fibroblasts were incubated with cell-free retroviral supernatants containing 5 μg/ml polybrene (Sigma Chemical Company, St Louis, Mo.) as previously described [22]. Ten days later the cells were harvested by trypsinization and inoculated subcutaneously into nu/nu recipients to select for tumorigenic cells. Tumors were excised, enzymatically digested in a mixture of dispase and collagenase as previously described [35], and transformed cell lines re-established *in vitro* by culturing and expanding the enzymatically disaggregated tumor cell suspensions in Dulbecco's minimum essential medium (DMEM; Stem Cell Technologies Inc.) containing 10% FCS (DMEM/10% FCS).

Monoclonal antibodies (mAb)

Hybridomas secreting mAb directed against the α subunit of Mac-1/CD11b (TIB128) [21] and the F4/80 antigen (HB198) [1] were obtained from ATCC. mAb 114/A10, directed against a 150-kDa antigen expressed on interleukin-3-dependent mast cell lines, primitive hemopoietic cells and mature tumor-associated macrophages, has been described in detail [12, 13, 24]. All hybridomas were maintained in DMEM/10% FCS and tissue-culture supernatants obtained as previously described [19].

Expression vectors

The pGEM2MCDF10 plasmid, containing a 4-kb murine M-CSF cDNA isolated from the pre-B-lymphocyte cell line 70Z/3 [26], was obtained from ATCC. A *Dra*III-*Sac*I fragment containing the entire M-CSF coding region but lacking extensive 5′ and 3′ untranslated sequences was isolated; the ends were blunted with T4 DNA polymerase, and the fragment obtained ligated into the *Hin*cII site of pUC13/18. Following digestion with *Xba*I, the fragment obtained was ligated into the *Xba*I site of the episomal expression vector pCDM8 [42] generating the plasmid pCDM8.mM-CSF. pCDM8 contains both SV40 and polyoma origins of replication and can replicate to high copy number within the nucleus of both SV40-transformed simian cell lines (e.g. COS7 cells) and polyoma-transformed murine cell lines (e.g. op/opPy) [42].

pRC3.mGM-CSF is a pCDM8-based expression vector containing the full-length murine granulocyte/macrophage colony stimulating factor (GM-CSF) cDNA. Details of its construction and characterization will be published elsewhere (manuscript in preparation).

Preparation of conditioned media (CM)

COS7 cells and op/opPy cells were transfected with plasmid DNA by electroporation using the BioRad Gene-Pulsar System (BioRad Laboratories Ltd., Mississauga, Ont.). Briefly, cells were trypsinized and resuspended in phosphate-buffered saline (PBS) at a final concentration of 5×10^6 cells/ml. Aliquots of 0.4 ml were mixed with approximately 2.5 μg plasmid DNA (pCDM8, pCDM8.mM-CSF or pCDM8.mGM-CSF), transferred to a 0.4-cm cuvette and electroporated at 280 V with a capacitance setting of 250 μF. The time constants obtained ranged from 6.5 ms to 7.4 ms. After electroporation, cells were incubated on ice for 5 min then diluted in 30 ml DMEM/10% FCS and plated in a 15-cm tissue-culture dish (Falcon 3025; Becton Dickinson). Supernatants were removed 72 h later, centrifuged at 250 *g* for 10 min to remove cellular debris, filtered through a 0.45 nicron filter (Syrfil-MF; Costar Corp., Cambridge, Mass.) and stored at −20 °C until required. Aliquots were thawed only once and any unused material discarded.

To obtain op/op, op/+, op/opPy and op/+Py conditioned media, 10-cm tissue-culture dishes (Falcon 3003; Becton Dickinson) were seeded with 1×10^6 cells in 10 ml DMEM/10% FCS. Cultures were incubated for 48 h at 37 °C, and the supernatants collected, processed and stored as described above.

Reverse transcriptase/polymerase chain reaction (RT-PCR) analysis of M-CSF transcripts

cDNA was prepared from 5 μg total RNA isolated from cell lines using the Pharmacia first-strand synthesis kit (Pharmacia, Baie d'Urfe, Quebec). Subsequent PCR was carried out exactly as described by the manufacturer using the following primer pair: 5′ M-CSF (5′-GCTCTAGAGCTGCCCGTATGACCGCGCG-3′) and 3′ M-CSF (5′-GCTCTAGAGGGGGTGTTGTCTTTAAAGC-3′). Both primers contain added 5′ *Xba*I restriction sites. The samples were placed in a thermal cycler (Biosycler, BIOS, New Haven, Conn.) and cycled 30 times at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. PCR products were separated on a 1% agarose gel, purified using a Geneclean II kit (BIO 101 Inc., Vista, Calif.), digested with *Xba*I and ligated into the *Xba*I site of pUC19. Double-stranded templates were sequenced by the dideoxy-DNA-chain-termination method [20], using T7 DNA polymerase and the reaction conditions suggested by the manufacturer (Sequenase Version 2.0 DNA sequencing kit, USB, Cleveland, Ohio).

Macrophage colony assay

Peripheral blood was collected from the inferior vena cava of anesthetized C3H/HeJ mice using a 1-ml syringe fitted with a 21-gauge needle. Sodium heparin (Stem Cell Technologies Inc.) was added to

a final concentration of 10 U/ml to prevent clotting. The blood was diluted 1:4 in PBS and 8 ml gently layered over 4 ml Lympholyte M (Cedarlane, Hornby, Ont.) in a 15-ml conical centrifuge tube (Falcon 2096; Becton Dickinson). Tubes were centrifuged at 300 *g* for 20 min at room temperature and the peripheral blood leukocytes (PBL) located at the blood/Lympholyte M interface collected, washed once with Hank's balanced salt solution (HBSS) and resuspended in alpha modified Eagle's medium (αMEM; Stem Cell Technologies Inc.) containing 20% fetal clone I (FCI; Hyclone) (αMEM/20% FCI).

Samples comprising 1×10^4 PBL together with dilutions of conditioned medium (see above) were added in a final volume of 1 ml α MEM/20% FCI to the wells of a 24-well plate (Falcon 3047; Becton Dickinson). The plates were incubated for 10–12 days at 37 °C, and the number of macrophage colonies, each derived from a single macrophage progenitor, were quantified by washing the wells twice with PBS and then staining the adherent cells for approximately 10 min with 1% methylene blue in methanol. Colonies containing more than 10 cells were counted using a inverted-phase microscope.

In order to confirm that the cells present within these adherent colonies were indeed macrophages, representative wells were harvested by incubation with PBS containing 2 mM EDTA and cytospin preparations prepared and stained with a panel of mAb using an indirect immunoperoxidase technique, as described below.

Generation of op/opPy and op/+Py tumors

To produce experimental tumors with which to investigate the role played by tumor-cell-derived M-CSF in regulating the recruitment and phenotype of tumor-associated macrophages, 1×10^6 in vitro cultured op/opPy or op/+Py cells, harvested by trypsinization, were injected subcutaneously into the flank of SCID mice.

Indirect immunoperoxidase staining

Indirect immunoperoxidase staining was used to determine the number of macrophages present within enzymatically disaggregated tumor cell suspensions. Briefly, cytospin preparations were air-dried, fixed in acetone for 5 min, and incubated for 30 min at room temperature with 50 µl of the appropriate mAb tissue-culture supernatant. After extensive washing in HBSS, the cytospins were incubated for a further 30 min with 50 µl of a 1:2000 dilution of horseradish-peroxidase-conjugated goat anti-(rat IgG) (Sigma) (TIB128, HB198, 114/A10). After washing in HBSS, the reaction was developed by incubating the slides for 5 min at room temperature in PBS containing 0.06% (w/v) 3-3′-diaminobenzidine (Sigma) and 0.012% (v/v) H_2O_2 (Sigma).

Northern blot analysis

Total cellular RNA was isolated from parental and transformed op/op and op/+ cell lines and enzymatically disaggregated tumor cell suspensions, using the guanidine isothiocyanate/CsCl method as previously described [10]. Samples containing 10 µg RNA were electrophoresed through a 1% (w/v) agarose gel containing 5% (v/v) de-ionized formaldehyde, transferred to a nylon membrane (Zeta-Probe GT; BioRad) and cross-linked by exposure to ultraviolet radiation (Stratalinker; Stratagene, La Jolla, Calif.). Filters were pre-hybridized for 1 h at 42 °C in 500 mM sodium phosphate buffer, pH 7.2, 50% (v/v) formamide, 5% sodium dodecyl sulfate, 1 mM EDTA, and 1 mg/ml bovine serum albumin (Fraction V; Sigma) and then hybridised for 16 h at 42 °C in the same solution containing denatured ^{32}P-labeled probes prepared using the Ready-To-Go oligonucleotide labelling kit according to the manufacturer's instructions (Pharmacia). Filters were washed and developed as previously described [23].

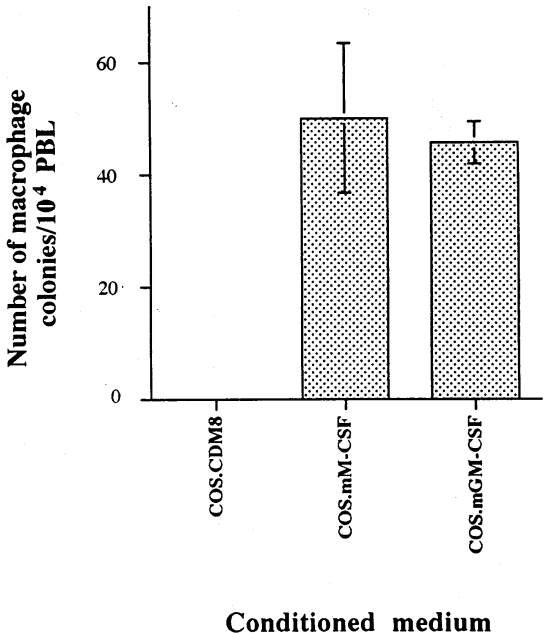

Fig. 1 Requirement for macrophage-colony-stimulating factor (*M-CSF*) or granulocyte/macrophage-colony-stimulating factor (*GM-CSF*) in the proliferation and differentiation of macrophage colonies. Murine peripheral blood leukocytes (*PBL*) were incubated with α modified Eagle's medium/10% fetal clone I containing 10% COS.CDM8, COS.mM-CSF or COS.mGM-CSF conditioned medium and the number of macrophage colonies generated was determined on days 10–12 following fixation and staining in a solution of 1% methylene blue in methanol. Results shown represent the means of three experiments ± SEM

Results

Characterization of op/op and op/+ fibrobalastoid cell lines

As shown in Fig. 1, the proliferation and differentiation of peripheral-blood-derived macrophage colonies in vitro is critically dependent upon the presence of appropriate hemopoietic growth factors. Thus, medium conditioned by COS7 cells transfected with the pCDM8 expression vector alone was unable to support the generation of significant numbers of macrophage colonies. In contrast, medium conditioned by COS7 cells transfected with pCDM8.M-CSF or pRC3.GM-CSF induced large numbers of adherent macrophage colonies which, by day 14, contained 20–200 cells. Although there are clear differences in the morphology of the adherent cells present within the colonies induced by M-CSF and GM-CSF in both instances, more than 95% of the cells produced are $CD18^+$, $CD44^+$ and $CD45^+$ and express the macrophage markers Mac-1 (CD11 b) and F4/80 (data not shown).

Media conditioned by fibroblastoid cell lines derived from op/op mice and their phenotypically normal littermate controls were also tested for their supportive capacity. Although both cell lines expressed approximately equal levels of M-CSF mRNA (Fig. 2), only medium conditioned by fibroblasts derived from phenotypically normal mice

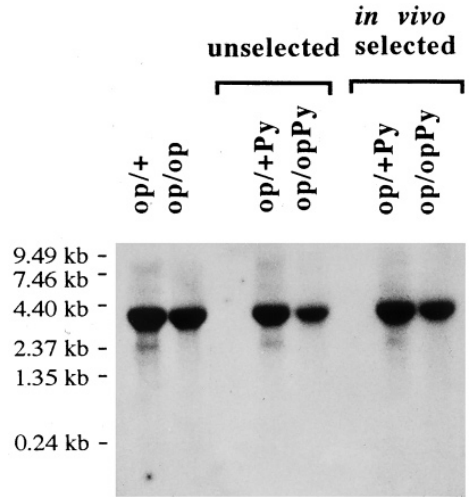

Fig. 2 Northern blot analysis of M-CSF mRNA expression in normal and transformed op/opPy− and op/+Py-derived fibroblastoid cell lines. Samples comprising 10 μg total cellular RNA isolated from op/op and op/+, unselected CTV-4Py-transformed and tumorigenic in-vivo-selected op/opPy and op/+Py cells were run in each lane and filters hybridised with a ^{32}P-labeled cDNA probe encompassing the entire coding region of the murine M-CSF gene. The result shown is representative of the data obtained in three independent experiments

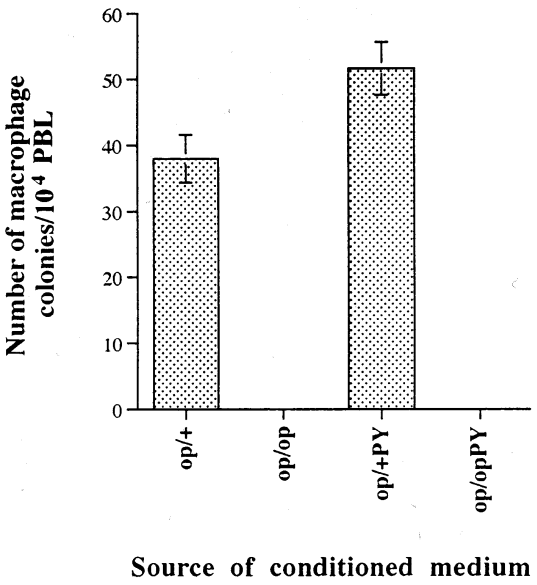

Fig. 3 M-CSF production by op/op, op/+, op/opPy and op/+Py cells. Samples comprising 1×10^4 PBL were incubated with 10% conditioned medium from op/op, op/+, op/opPy and op/+Py cells. The number of macrophage colonies containing more than 10 cells was determined on days 10−12 following fixation and staining in a solution of 1% methylene blue in methanol. The results shown here represent the mean ± SEM from three separate experiments

was able to support the generation of macrophage colonies in vitro from peripheral blood precursor cells (Fig. 3). Medium conditioned by the op/op cell line had essentially no supportive activity, even when tested at a high concentration.

In agreement with previous reports, sequence analysis of RT-PCR products demonstrated that the M-CSF gene present within the op/op cell line contained an additional thymidine residue 262 bp downstream of the ATG start codon (Fig. 4). The presence of this additional nucleotide produces a shift in reading frame that generates a premature stop codon 27 bp downstream of the point of insertion (Fig. 4). Similar RT-PCR analysis of the cell line derived from phenotypically normal littermates revealed the presence of both wild-type and mutant M-CSF transcripts (Fig. 4), indicating that at least one of the animals used to generate this line was a heterozygote (op/+). Taken together, these data confirm that the op/op fibroblastoid cell line utilized in this study was deficient in its production of biologically active M-CSF.

Generation and characterization of polyoma-transformed op/op and op/+ cell lines

To generate tumorigenic cell lines that could be used to determine the role played by tumor-cell-derived M-CSF in regulating the accumulation and/or phenotype of tumor-associated macrophages, subconfluent monolayers of op/op and op/+ fibroblasts were infected with an ecotropic retroviral vector, designated CTV-4Py, that encodes the polyoma large T antigen. Ten days later, numerous foci (more than 100) of transformed cells were evident in dishes infected with the CTV-4Py vector but not in equivalent dishes infected with the control CTV-4 vector lacking polyoma-derived sequences (data not shown). The cells from infected and control cultures were then harvested and inoculated subcutaneously into BALB/c-nu/nu recipients (3 mice/group). nu/nu recipients were used to avoid rejection of tumor cells expressing highly antigenic polyoma-derived proteins. By day 40, tumors had developed in all of the mice that had received CTV-4Py-infected op/op or op/+ fibroblasts but not in any of the mice that had received control CTV-4-infected cells (data not shown).

The tumors that developed were excised, enzymatically disaggregated and polyoma-transformed op/op (op/opPy) and op/+ (op/+Py) cell lines re-established in vitro. Northern blot analysis and indirect immunoperoxidase staining confirmed that both of these cell lines, but not the parental (non-transformed) op/op and op/+ cell lines, express polyoma large T antigen (data not shown).

To confirm that the op/opPy cell line remains deficient in its production of M-CSF, op/opPy and op/+Py cells were also tested for their ability to induce the proliferation of macrophage colonies in vitro. Although Northern blot analysis indicated that both cell lines express approximately equal levels of M-CSF mRNA (Fig. 2), only medium conditioned by the op/+Py cell line was able to induce the generation of macrophage colonies from peripheral blood leukocytes (Fig. 3).

To confirm that the major reason for the inability of the op/opPy cells to induce the proliferation of peripheral blood-derived macrophage precursors was the absence of

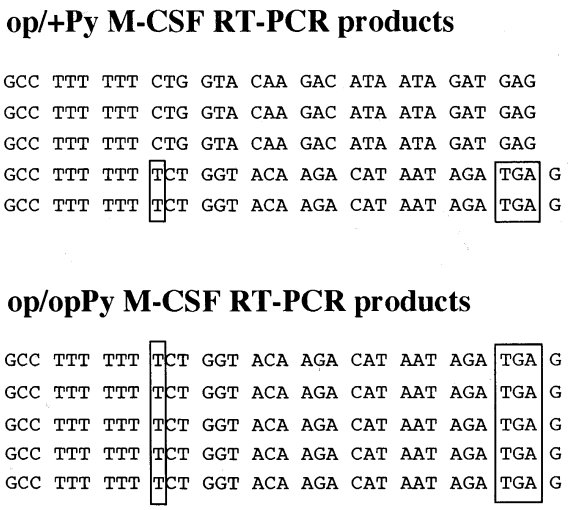

Fig. 4 Reverse transcriptase/polymerase chain reaction (*RT-PCR*) analysis of M-CSF transcripts in fibroblastoid cell lines derived from op/op mice and phenotypically normal littermates. M-CSF cDNA were amplified by RT-PCR and subcloned into pUC19; individual clones were sequenced

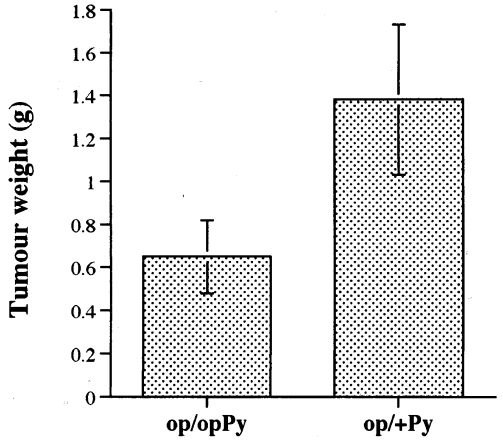

Fig. 6 Growth of op/opPy and op/+Py tumors. Samples containing 1×10^6 op/opPy and op/+Py cells were inoculated subcutaneously into the flank of groups of at least 5 BALB/c SCID mice. The weight of the tumors produced was determined on day 28. op/+Py tumors were significantly larger than op/opPy tumors ($p<0.05$, Student's *t*-test). The result shown is representative of the data obtained in at least four independent experiments

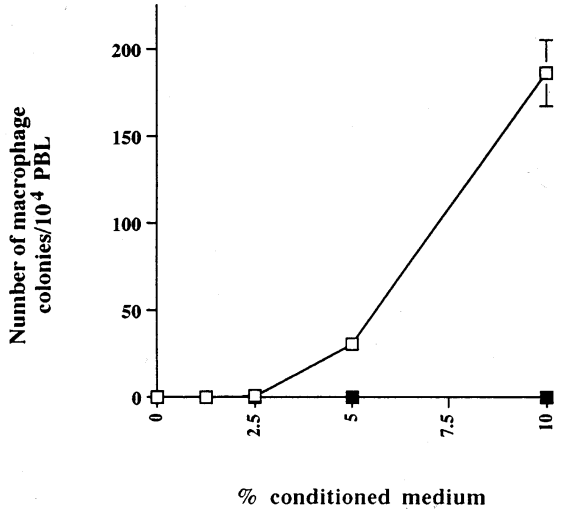

Fig. 5 M-CSF production by pCDM8- and pCDM8.mM-CSF-transfected op/opPy cells. Samples comprising 1×10^4 PBL were incubated with various concentrations of medium conditioned by pCDM8- or pCDM8.mM-CSF-transfected op/opPy cells. The number of macrophage colonies containing more than 10 cells was determined on days 10–12 following fixation and staining in a solution of 1% methylene blue in methanol

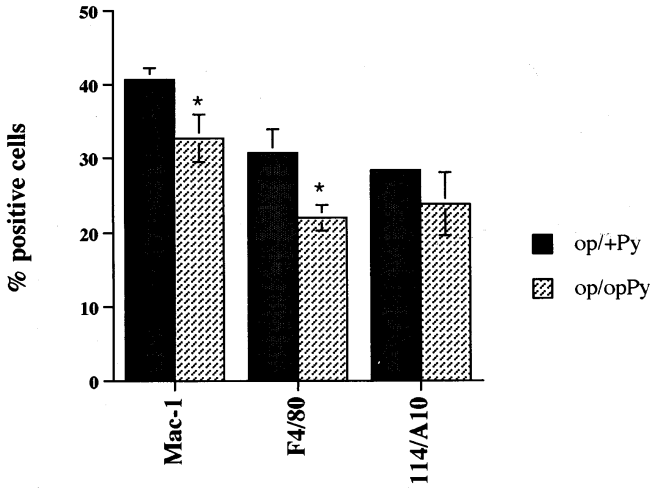

Fig. 7 Macrophage content of op/opPy and op/+Py tumors. Acetone-fixed cytospin preparations of enzymatically disaggregated tumor cell suspensions were stained with mAb directed against the α subunit of Mac-1 (TIB128), the F4/80 antigen (HB198) and 114/A10 using an indirect immunoperoxidase technique. Each point represents the mean ± SEM of at least 3 experiments in which tumors were pooled from groups of 3 or more mice. On each cytospin at least 200 cells were counted. The percentage of Mac-1-positive and F4/80-positive cells present in op/opPy tumors was significantly lower ($p<0.05$) than in op/+Py tumors. There was no significant difference in the percentage of 114/A10-positive cells ($p = 0.14$). *p* values were obtained using Student's *t*-test

functional M-CSF, and to rule out other possibilities, such as the production by these cells of soluble mediators that block the action of M-CSF or other hemopoietic growth factors, op/opPy cells were transfected with a pCDM8-based episomal expression vector encoding murine M-CSF, and supernatants conditioned by these cells or by control pCDM8-transfected cells were tested for their ability to support the proliferation and/or differentiation of macrophage precursors in vitro. As shown in Fig. 5, medium conditioned by op/opPy cells transfected with pCDM8.M-CSF was fully capable of generating large numbers of macrophage colonies. Control supernatants conditioned by op/opPy cells transfected with the pCDM8 vector alone had no supportive capacity.

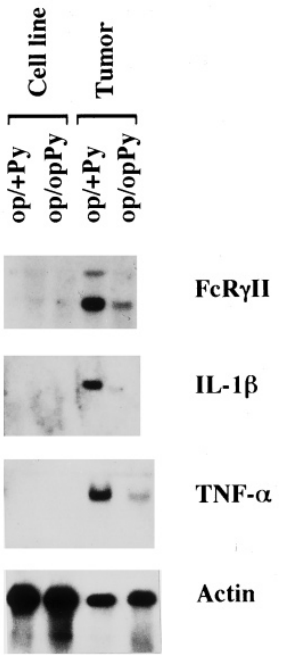

Fig. 8 Northern blot analysis of FcRγII, interleukin-1β (*IL-1β*) and tumor necrosis factor α (*TNFα*) mRNA expression in op/opPy and op/+Py tumor-associated macrophages. Samples of 10 μg total cellular RNA were run in each lane. Filters were hybridised with ^{32}P-labeled probes encompassing the entire coding regions of murine FcRγII, IL-1β, TNFα and actin. The result shown is representative of the data obtained in three independent experiments

Role of tumor-cell-derived M-CSF in regulating the accumulation and phenotype of tumor-associated macrophages

In order to define the role of tumor-cell-derived M-CSF in regulating the accumulation and phenotype of tumor-associated macrophages, 1×10^6 op/opPy or op/+Py cells were inoculated subcutaneously into SCID mice and both the size and weight of the tumors generated and their macrophage content were determined on day 28. As shown in Fig. 6, op/+Py tumors were significantly larger than the equivalent op/opPy tumors. The macrophage content of op/opPy or op/+Py tumors was determined by indirect immunoperoxidase staining of cytospin preparations of enzymatically disaggregated tumor cell suspensions using a panel of anti-macrophage mAb. As shown in Fig. 7, although the proportion of Mac-1-positive and F4/80-positive cells is significantly higher in op/+Py tumors, both tumor types contain large numbers of macrophages and the proportion of 114/A10-positive cells was remarkably similar. Despite the fact that small day-10 tumors contain a somewhat lower percentage of F4/80-positive cells than the large day -28 tumors, the difference between op/+Py and op/opPy, while once again modest, is still significant (op/+Py 21.7+1.6%: op/opPy 16.3+1.8%: $p<0.05$). Interestingly, however, the macrophages, present within op/opPy and op/+Py tumors exhibited obvious morphological differences, with those present in op/opPy tumors appearing smaller and less mature (data not shown), suggesting that, although M-CSF may not play a major role in regulating the accumulation of tumor-associated macrophages, it does nevertheless, have an effect on the phenotype of these cells. Indeed, in support of such a role, Northern blot analysis demonstrated that the macrophages found within op/opPy tumors expressed far lower levels of lL-1β and TNF-α and FcRγII mRNA than did equivalent cells isolated from op/+Py tumors (Fig. 8).

Discussion

While it is generally agreed that the overall macrophage content of a tumor is determined both by the recruitment of monocytes from the circulation and the local proliferation and differentiation of macrophage progenitor cells within the tumor microenvironment [14, 30, 33], at present, neither the relative importance of each of these mechanisms, nor the precise molecular events involved have been adequately defined. What is clear is that, while different experimental tumors may vary greatly in their macrophage content [14], the percentage of macrophages present within a particular tumor remains fairly constant both during periods of logarithmic growth and upon transplantation to secondary recipients [14, 30, 33]. Moreover, metastatic tumor deposits frequently contain the same proportion of macrophages as the primary tumors from which they were derived [47]. Taken together, these findings suggest that signals derived from tumor cells themselves, rather than tumor-associated tissue-specific stromal elements, are primarily responsible for determining the macrophage content of a particular tumor.

Several tumor-cell-derived factors chemotactic for monocytes have recently been identified [7, 32, 39, 46, 52] and shown to play an important role in regulating the accumulation of tumor-associated macrophages [4–6, 8, 30, 40, 47]. These include MCP-1, MCP-2, MCP-3, and VEGF/VPF. There is also evidence that these are not the only molecules involved in this complex process [30].

The major aim of the present study was to determine what role, if any, tumor-cell derived M-CSF may play in determining the total macrophage content of a tumor. M-CSF is an attractive candidate in this regard. It is constitutively produced at a high level by many histologically distinct human and animal tumors [31] and has been shown to function both as a monocyte-specific chemoattractant [48], and to promote the survival, proliferation and differentiation of monocytes and other macrophage precursor cells in vitro [45] and in vivo [6, 9]. The results of the present study, however, demonstrate that tumors initiated from M-CSF-deficient op/opPy fibroblastoid cells contain almost as many macrophages as similar tumors initiated from M-CSF-producing op/+Py cells. These findings argue against a prerequisite role of M-CSF for the accumulation of macrophages within at least this tumor.

This conclusion contrasts with those of recent studies, in which the introduction of the human M-CSF gene into the murine plasmacytoma cell line J558L was shown to in-

crease dramactically the number of macrophages present within tumors generated from these cells following inoculation into syngeneic BALB/c mice [11]. Growth of these M-CSF-transduced tumors was only slightly inhibited, and attempts to activate the tumor-associated macrophages by the systemic administration lipopolysaccharide and/or interferon γ (IFNγ) were unsuccessful. Although the reason for the difference between these results and the data presented here remains to be determined, they may simply reflect the fact that the very high and non-physiological level of M-CSF produced by transfected J588L cells is sufficient to induce dramatic increases in both the number and functional activity of circulating monocyte/macrophages in tumor-bearing animals [11].

Although the molecular nature of the molecule(s) that can maintain monocyte viability within M-CSF-deficient op/opPy tumors remains to be determined, there are several obvious possibilities. Firstly, host-derived stromal elements or immune cells could perhaps provide sufficient amounts of M-CSF to maintain monocyte/macrophage viability. Arguing against this possibility, however, is the observation, discussed further below, that macrophages present within tumors initiated from op/opPy cells express far lower levels of several M-CSF-inducible mRNA transcripts, including IL-1β, TNFα and FcRγII, than equivalent cells present within op/+Py tumors. Thus, if M-CSF is produced within op/opPy tumors, it must be assumed that the levels generated, while sufficient to maintain monocyte/macrophage viability at a level similar to that seen in op/+Py tumors, are nevertheless insufficient to induce equivalent levels of IL-1β, TNFα and FcRγII gene expression within these cells.

GM-CSF can also promote monocyte survival in vitro. Although most fibroblastoid cell lines do not constitutively produce GM-CSF, many can be induced to do so following stimulation with the macrophage-derived cytokines IL-1β, TNFα or IFNγ. Arguing against a role for this molecule in the accumulation of tumor-associated macrophages, however, was the finding that GM-CSF was not constitutively produced at detectable levels by either op/opPy or op/+Py cells, nor could these cells be readily induced to secrete this cytokine following stimulation with IL-1β or TNFα (data not shown). Moreover, Northern blot analysis failed to demonstrate the presence of significant quantities of GM-CSF mRNA within $CD45^-/CD18^-$ tumor cells purified from either op/+Py or op/opPy tumors (data not shown).

A final possibility is that op/opPy cells may elaborate a soluble mediator distinct from M-CSF or GM-CSF that can promote monocyte survival in vitro. Evidence supporting the existence of such a molecule can be inferred from the observation that mice deficient in both M-CSF and GM-CSF still contain significant numbers of circulating monocytes and near-normal numbers of macrophages in at least certain tissues [28].

Although tumor-cell-derived M-CSF does not appear to play a critical role in regulating the accumulation of tumor-associated macrophages, it can have an effect on the phenotype of these cells. This conclusion is based on the finding that macrophages present within op/opPy tumors were morphologically distinct, and expressed lower levels of IL-1β, TNFα and FcRγII mRNA than those present within op/opPy tumors. All three of these latter genes have been shown previously to be induced by M-CSF in vitro. In addition, op/+Py tumors also appeared to grow somewhat faster than op/opPy tumors initiated with the same number of cells. How differences in tumor growth relate to the altered functional capabilities of the macrophages present within these tumors remains to be determined. It is interesting to note, however, that TNFα, which is expressed at a higher level within the faster growing op/+Py tumors, has been suggested to play an important role in the induction of tumor angiogenesis in vivo [16, 17, 24, 27, 34, 44].

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