The Detection and Localization of Monocyte Chemoattractant Protein-1 (MCP-1) in Human Ovarian Cancer

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Abstract

Chemokines may control the macrophage infiltrate found in many solid tumors. In human ovarian cancer, in situ hybridization detected mRNA for the macrophage chemokine monocyte chemoattractant protein-1 (MCP-1) in 16/17 serous carcinomas, 4/4 mucinous carcinomas, 2/2 endometrioid carcinomas, and 1/3 borderline tumors. In serous tumors, mRNA expression mainly localized to the epithelial areas, as did immunoreactive MCP-1 protein. In the other tumors, both stromal and epithelial expression were seen. All tumors contained variable numbers of cells positive for the macrophage marker CD68. MCP-1 mRNA was also detected in the stroma of 5/5 normal ovaries. RT-PCR demonstrated mRNA for MCP-1 in 7/7 serous carcinomas and 6/6 ovarian cancer cell lines. MCP-1 protein was detected by ELISA in ascites from patients with ovarian cancer (mean 4.28 ng/ml) and was produced primarily by the cancer cells. Human MCP-1 protein was also detected in culture supernatants from cell lines and in ascites from human ovarian tumor xenografts which induce a peritoneal monocytosis in nude mice. We conclude that the macrophage chemoattractant MCP-1 is produced by epithelial ovarian cancer and that the tumor cells themselves are probably a major source. MCP-1 may contribute to the accumulation of tumor-associated macrophages, which may subsequently influence tumor behavior. (*J. Clin. Invest.* 1995. 95:2391–2396.) Key words: chemokine • epithelial • macrophage • in situ hybridization • ascites

Introduction

Leukocytes infiltrate many solid malignant tumors (1). In human epithelial ovarian cancer, the infiltrating cells are predominantly macrophages and T lymphocytes (2). Tumor-associated macrophages (TAM)[1] are found both within the stromal (3) and epithelial tumor areas (4).

Although TAM may be cytotoxic to tumor cells when activated in vivo (5) or in vitro (6), the evidence that they normally have a tumoricidal role is scant. Indeed TAM may promote tumorigenesis by the production of cytokines such as EGF, PDGF, TGF-β, TNF-α, IL-1, and IL-6 (7). TNF-α can alter the behavior of ovarian cancer xenografts, causing tumor cells in ascites to form solid peritoneal tumors with well developed stroma (8). In situ hybridization studies show that TNF-α is expressed both by infiltrating macrophages and tumor epithelial cells, although TNF-α protein is largely restricted to TAM (4). TNF-α derived from TAM may also stimulate angiogenesis (9, 10) and TAM may contribute to tumor spread by producing matrix metalloproteases (11, 12).

A variety of human and murine tumor cells produce monocyte chemoattractant factors (13, 14) and there is a correlation between the amount of activity in culture supernatants and the number of TAMs when these cells produce tumors in vivo (15). Certain tumors, such as freshly disaggregated ovarian carcinomas, primary ovarian carcinoma cultures, and established ovarian carcinoma cell lines, produce a protein with an apparent molecular mass of 12 kD (16) which has chemotactic activity for peripheral blood monocytes. A protein, designated monocyte chemoattractant protein-1 (MCP-1), which has very similar properties, has been purified to homogeneity and the cDNA cloned from the glioma cell line U-105MG (17), the myelomonocytic lines HL60 and THP-1 (18, 19), and a variety of normal and transformed cells (20).

The aim of this study was to discover whether MCP-1 is expressed in human ovarian cancer, to establish its cellular sources in vivo and to determine whether there is a correlation between MCP-1 levels and the number of TAMs present. These findings were related to the in vitro production of MCP-1 by ovarian cancer cell lines and the production of MCP-1 by two human ovarian cancer xenografts that induced a monocytosis in their nude mouse hosts.

Methods

Tissue samples. Biopsy specimens of ovarian tumors from untreated patients were obtained at operation and snap frozen into liquid nitrogen.

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1. *Abbreviations used in this paper:* MCP, monocyte chemoattractant protein; RT, reverse transcription; TAM, tumor-associated macrophage.

They were then stored at −70°C before cutting 5–7-μm sections. Sections for in situ hybridization were mounted on baked glass slides coated with 3-aminopropyl-triethoxy-silane (Sigma Chemicals Ltd., Poole, Dorset, United Kingdom), air dried, and stored at −70°C until use. Sections for immunohistochemistry were cut onto poly-L-lysine–coated slides and stored at −70°C.

Cell lines. The following cell lines were used to prepare cDNA for reverse transcription (RT)-PCR: PEO 1, PEO 4, PEO 14, and PEA 2 (obtained from S. Langdon, Imperial Cancer Research Fund Oncology Unit, Western General Hospital, Edinburgh, United Kingdom), SKOV-3 (American Type Culture Collection, Rockville, MD), and OVCAR-3 (obtained from T. Hamilton, National Cancer Institute, Bethesda, MD). These lines grow as adherent monolayers. PEO 1, PEO 4, PEO 14, and PEA 2 were grown in RPMI 1640 medium supplemented with 10% FCS. OVCAR-3 was also grown in RPMI 1640, but in the presence of 10 μg/ml insulin. E4 was substituted for RPMI 1640 when culturing SKOV-3 cells. All cell lines were cultured in pyrogen-free conditions and in an atmosphere humidified with 5% CO_2 in air. The human promyelocytic cell line HL-60 (American Type Culture Collection) was used to provide positive controls for in situ hybridization and RT-PCR. HL60 cells were maintained in RPMI 1640 supplemented with 5% FCS and were stimulated with 10 ng/ml human recombinant TNF-α for 6 h before preparing cytospins or RNA.

Mice and xenografts. 6–12-wk-old specific pathogen-free female nu/nu (nude) mice of mixed genetic background were maintained as described previously (21). The ovarian cancer xenografts LA and HU were established from primary human tumors as described previously (22). The ascites were confined to the peritoneum and were passaged in vivo. For protein estimation, the mice were injected intraperitoneally with 4 ml of RPMI 1640. Once removed, the ascites were spun and the supernatant was stored at −70°C.

Riboprobes. A template consisting of an ~360-bp fragment of MCP-1 cDNA in the expression vector pGEM 3Z (Sigma Chemicals Ltd.) was linearized with BamH1 (Promega Corp., Southampton, United Kingdom) and transcribed with SP6 RNA polymerase (Promega Corp.) to generate antisense riboprobes. Sense probes were made using HindIII and T7 polymerase (Promega Corp.). Antisense β-actin (obtained from Dr. L. Kedes, Stanford University, Stanford, CA) was used as a positive control for all in situ hybridization experiments. Probes were generated from HindIII-cleaved Bluescript containing human β-actin cDNA using T7 RNA polymerase. Both sense and antisense MCP-1 riboprobes were used to probe TNF-α–stimulated HL60 cytospins.

In situ hybridization. The method described by Naylor et al. (23) was followed. In brief, cryostat sections mounted on 3-aminopropyl-triethoxy-silane–coated slides were fixed in 4% paraformaldehyde (Sigma Chemicals Ltd.) for 20 min, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated through an ethanol gradient, and air dried. 5×10^4 dpm RNA probe/μl was added to the hybridization mix. Hybridization was carried out overnight at 50°C. After hybridization, the slides were washed in dilutions of SSC and treated with 20 μg/ml RNase A, after which they were dehydrated through a graded alcohol series, air dried, and dipped in liquefied K5 emulsion (Ilford Ltd., Basildon, Essex, United Kingdom). The sections were exposed for 10 d at 4°C before being developed and counterstained with toluidine blue.

Immunohistochemistry. The avidin-biotin-peroxidase complex method was used to stain both for macrophages and MCP-1 protein (24). Sections were air dried overnight and fixed in 4% paraformaldehyde in PBS for 5 min before staining. They were preincubated with normal rabbit serum (DAKO, High Wycombe, Bucks, United Kingdom) diluted 1:25 for 15 min before the primary antibody was applied.

To visualize macrophages, sections were incubated for 30 min at room temperature with the anti-CD68 mAb EBM11 (DAKO) diluted 1:100. An mAb to MCP-1, 5D3-F7 (25) diluted 1:100, was used to localize MCP-1 protein. These sections were incubated overnight at 4°C. For both antibodies, sections were then incubated with biotinylated rabbit anti–mouse IgG (DAKO) and avidin-biotin-peroxidase complex (DAKO). The final incubation was with the chromogen 3,3′-diaminobenzidine tetra-hydrochloride, giving a brown reaction product. The sections were counterstained with hematoxylin (Sigma Chemicals Ltd.) and mounted in DPX (BDH Laboratory Supplies, Merck Ltd., Lutterworth, United Kingdom).

Preparation of RNA from tissue samples and cell lines. Total RNA was prepared from human ovarian tissue by the method of Chomczynski (26). RNA was quantitated by absorbance at 260 nm and the samples were treated with DNase before being used for cDNA synthesis.

Oligonucleotide primers. The MCP-1 primers used were as follows: 5′-CAAACTGAAGCTCGCACTCTCGCC -3′ (−30 to −6) and 5′-ATTCTTGGGTTGTGGAGTGAGTGTTCA-3′ (299–324) giving a PCR product of 354 bp. For β-actin, used as a control, the primers were: 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTT-3′ yielding a product of 548 bp. The MCP-1 sequences were chosen to span the protein coding region within the MCP-1 cDNA PstI restriction sites.

RT-PCR. cDNA was prepared using the methods and reagents of the Boehringer Mannheim cDNA strand synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany) for the first strand synthesis only. PCR was carried out using the Perkin-Elmer Gene Amp PCR Kit (Perkin-Elmer Corp., Norwalk, CT), using MCP-1 or β-actin specific primers. A reaction volume of 25 μl per sample was made up. PCR was carried out on 2-μl aliquots of cDNA using 35 amplification cycles. Amplification products were electrophoresed through a 1.2% agarose gel and visualized by ethidium bromide staining.

Characteristics of patients used in analysis of ascites. Ascites were collected by paracentesis from a total of 46 patients with ovarian cancer. 30 patients were subsequently shown histologically to have serous adenocarcinoma, 3 mucinous adenocarcinoma, 5 clear cell carcinoma, 4 endometrioid carcinoma, 3 undifferentiated carcinoma, and 1 an anaplastic mucous-secreting tumor. 20 patients had received some treatment before their ascites were analyzed and 26 were untreated.

Isolation of TAM and ovarian carcinoma cells. TAM and ovarian carcinoma cells were separated from ascites using discontinuous Ficoll-Hypaque and Percoll gradients, essentially as described (16). Highly enriched TAM preparations (> 95% by morphology) were obtained using CD14-coated magnetic beads (Unipath, Milan, Italy). Tumor cell preparations were depleted of $CD14^+$ cells. TAM and tumor cells were cultured at 3×10^6/ml in RPMI 1640 with 10% FCS. After 24 h supernatants were collected for MCP-1 measurement.

ELISA for MCP-1 in ascites, TAM and tumor cell preparations, tumor cell lines and xenografts. MCP-1 was measured using a sandwich ELISA assay based on a rabbit antiserum and an mAb (5D3-F7), as described recently (25). The assay has a sensitivity of 40 pg/ml in supernatants and 80 pg/ml in peritoneal fluids. It is specific for human MCP-1 and does not detect the mouse equivalent, JE, or the closely related human chemokines MCP-2 and MCP-3.

Results

MCP-1 mRNA and protein in human ovarian cancer biopsies. In situ hybridization demonstrated mRNA for MCP-1 in a total of 23/26 ovarian tumors (Table I). In the group of serous adenocarcinomas, mRNA expression was detected in 16/17 samples. In 13 of these cases it was possible to distinguish between stromal and epithelial expression; while some label was found at the stromal-epithelial border in all 13 cases, it was predominantly confined to the epithelium in 11/13 (Fig. 1, *A* and *B*) and to the stroma in 2/13 (Fig. 1 *C*). Within the epithelium, expression was confined to single cells or small groups of cells. The maximum expression was 12.4% epithelial cells/high power field and the mean was 1.4%. 4/4 mucinous tumors expressed mRNA; 1 expressed predominantly epithelial mRNA while in the other 3 it was confined to the stroma or stromal-epithelial interface. In 1/2 endometrioid carcinomas, expression of MCP-1 mRNA was equal between the stroma and the epithe-

Table I. Frequency and Extent of MCP-1 mRNA Expression in Ovarian Cancer Biopsies Determined by In Situ Hybridization

Tumor type	Number of cases	Total number positive	±	+	++
Serous	17	16	1	3	12
Mucinous	4	4	0	1	3
Borderline	3	1	0	0	1
Endometrioid	2	2	0	2	0
Nonmalignant ovarian tissue	5	5	1	1	3

±, < 0.2 cells/high power field; +, 0.2–2 cells/high power field; ++, 2–20 cells/high power field. The mean number of labeled cells in 10 high power fields was calculated for each sample.

lium and in 1/2 it was predominantly stromal. Only 1/3 of the borderline tumors expressed MCP-1 (Table II). In this case, localization of mRNA to individual cells within the epithelial monolayer was clear, but stromal mRNA was also present (Fig. 1 *D*). Approximately 20% of HL60 cells were positive for MCP-1 mRNA in control cytospins probed with antisense riboprobe. mRNA for MCP-1 was also detected by RT-PCR in 7/7 serous adenocarcinomas analyzed.

MCP-1 immunoreactivity was present in some serous tumors. In one case, staining was clearly confined to the tumor epithelium and was most prominent in the apical part of cells. In another poorly differentiated tumor, staining was again seen throughout the epithelium but was more homogeneously distributed within individual cells. In other cases, immunoreactivity was sporadic with only a small amount of perinuclear staining, but again this was confined to cells within the epithelium.

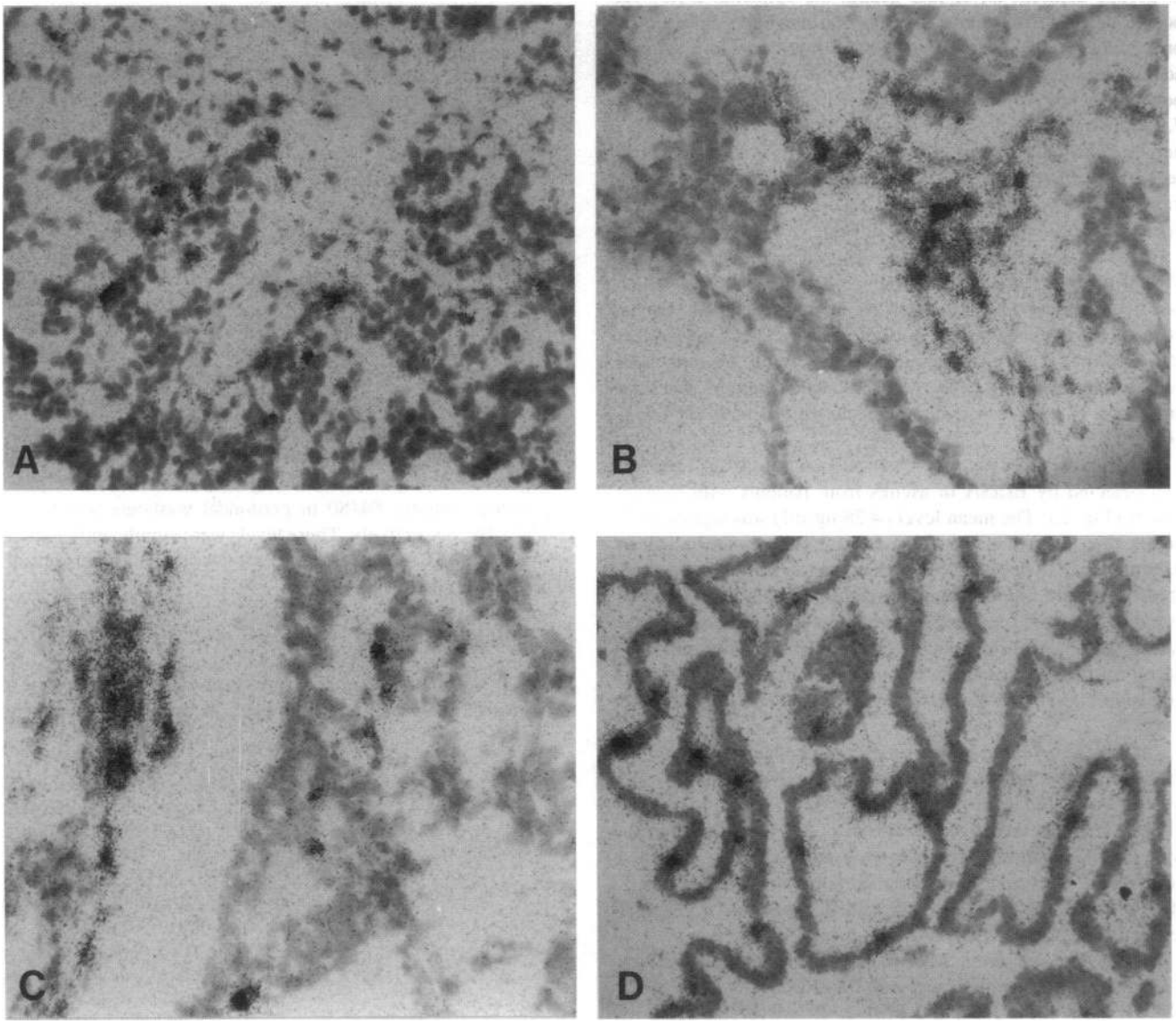

Figure 1. The distribution of MCP-1 mRNA and protein as determined by in situ hybridization with a ^{35}S-labeled antisense MCP-1 riboprobe. (*A*) Individual cells labeled within an epithelial area of a serous adenocarcinoma, × 200 and (*B*) × 650. (*C*) Stromal expression of MCP-1 mRNA in a serous adenocarcinoma, × 650. (*D*) Focal epithelial labeling in a serous borderline tumor, × 200.

Table II. Epithelial versus Stromal Localization of MCP-1 mRNA

	Predominantly epithelial	Predominantly stromal	Stroma and epithelium
Serous adenocarcinoma	11/13*	2/13	0/13
Mucinous adenocarcinoma	1/4	3/4	0/4
Endometrioid carcinoma	0/2	1/2	1/2
Borderline tumor	0/3	0/3	1/3
Normal ovary	0/5	5/5	0/5

* The tissue preservation in the other four cases was not sufficient for analysis of localization.

TAMs in human ovarian cancer. All the tumors studied contained cells which were positive for the macrophage marker CD68 (for review see Weiss et al. [27]), within both the stromal and epithelial areas. CD68-positive cells were mainly found in groups adjacent to, or just within, the epithelial areas, but occasionally, positive cells aggregated in small areas of necrosis or were observed in glandular lumina. In serous and mucinous adenocarcinomas the proportion of CD68 positive cells varied between 1 and 28% cells/high power field with a mean of 7.8%. However, there was no statistically significant correlation between the number of cells expressing MCP-1 mRNA in a section and the number of CD68-positive cells detected by immunohistochemistry (Mann-Whitney two-sample test and Spearman's rank correlation coefficient).

MCP-1 and macrophages in nonmalignant ovarian tissue. MCP-1 mRNA was detected in the stroma of 5/5 ovaries which had been removed for a variety of nonmalignant conditions. Stromal macrophages could be identified by CD68 staining in all cases, but it was not possible to determine whether these cells were expressing MCP-1 mRNA. Mesothelium was only preserved in one case and in this no epithelial mRNA was expressed.

MCP-1 levels in human serum and ascites. MCP-1 protein was detected by ELISA in ascites from patients with ovarian cancer (Fig. 2). The mean level (4.28 ng/ml) was significantly higher than that in ascites from patients with cirrhosis (mean 0.76 ng/ml; $P < 0.00001$ by Student's t test). Low but detectable levels were found in plasma from normal laboratory donors (mean 0.23 ng/ml), patients with benign gynecological disease (mean 0.39 ng/ml), and patients with ovarian carcinoma (0.49 ng/ml). In ovarian cancer patients, the ascites levels were significantly higher than plasma levels ($P < 0.00001$ by Student's t test).

MCP-1 production by freshly isolated TAM and tumor cells. In three patients highly enriched TAM and ovarian tumor cells were fractionated from ascites and their capacity to release MCP-1 was assessed over a 24-h period. MCP-1 production was essentially confined to the ovarian carcinoma–enriched population. The tumor cell–enriched population produced 2.3, 40.9, and 47.2 ng/ml compared with 0.3, 0.2, and 0.1 ng/ml, respectively, produced by TAM.

MCP-1 in cell lines. MCP-1 expression was detected by RT-PCR in the ovarian cancer cell lines SKOV-3, OVCAR-3, PEO 1, PEO 4, PEO 14, and PEA 2. This was confirmed by Southern blotting (Fig. 3). Ovarian cancer cell lines were heterogeneous in terms of MCP-1 protein production. PEO 14 produced the highest level (mean 6.77±0.01 ng/ml); low but

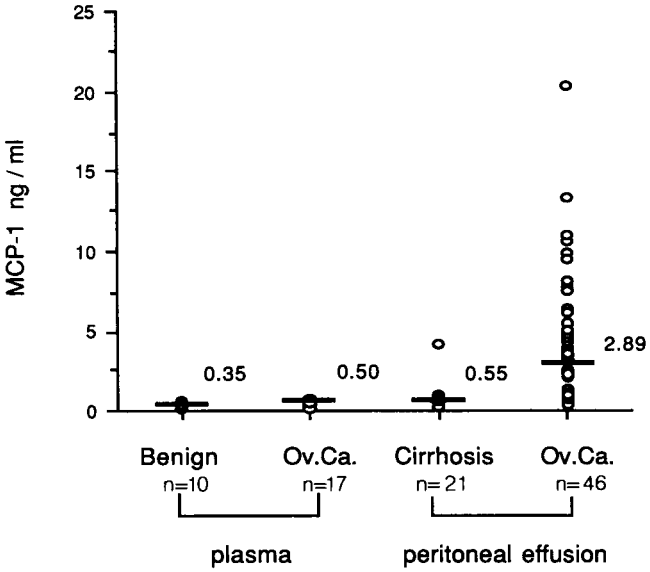

Figure 2. Plasma and ascites levels of MCP-1 determined by specific ELISA using the mAb 5D3-F7. The numbers next to each group of results refer to the median values of MCP-1 (nanograms per milliliter) detected. There is a significant difference ($P > 0.00001$) between the levels in ascites from patients with ovarian carcinoma and those with cirrhosis. The difference between plasma and ascites levels is also significant.

appreciable levels were found in PEO 1 (mean 0.11±0.01 ng/ml), PEO 4 (mean 0.27±0.01 ng/ml), and OVCAR-3 (mean 0.09±0.01 ng/ml), and little or no MCP-1 was detected in supernatants from PEA 2 (mean 0.04±0.01 ng/ml) and SKOV-3 (0.06±0.01 ng/ml).

Ovarian cancer xenografts. Human MCP-1 protein was detected in peritoneal washouts from both HU (mean 0.07±0.02 ng/ml) and LA (mean 0.38±0.01 ng/ml) ovarian cancer xenografts. 7 d after intraperitoneal injection of the HU and LA xenografts, the proportion of cells positive for the mouse pan-macrophage marker F4/80 in peritoneal washouts was 65±5 and 54±4%, respectively. These levels were significantly higher ($P < 0.005$ for HA and $P < 0.025$ for LA) than those in control mice (40±1.1%).

Discussion

Using in situ hybridization we have demonstrated that MCP-1 is expressed in human epithelial ovarian cancer. Furthermore,

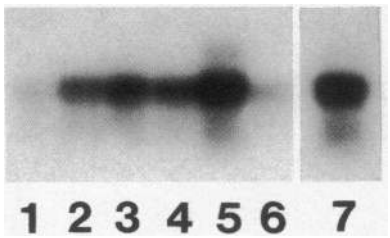

Figure 3. MCP-1 mRNA expression in ovarian cell lines. Lane *1*, SKOV-3; lane *2*, OVCAR-3; lane *3*, PEO 1; lane *4*, PEO 4; lane *5*, PEO 14; lane *6*, PEA 2; and lane *7*, HL60. A randomly primed [α-^{32}P]dCTP-labeled probe generated from MCP-1 cDNA was used to probe ovarian cell line PCR amplification products using MCP-1 specific primers. The autoradiograph was exposed for 5 min at room temperature.

MCP-1 protein is detectable in significant levels in ascites from these patients. Within the serous tumor group, MCP-1 mRNA is predominantly associated with the epithelial areas, although stromal expression was occasionally detected. Where immunoreactivity for MCP-1 was found, it was also within the epithelium, and when TAMs were separated from tumor cells in ascites, the tumor cells were the major source of MCP-1. In mucinous tumors, mRNA expression was predominantly associated with the stroma, while in endometrioid tumors it was distributed throughout both the epithelium and stroma. The single borderline tumor that was positive for MCP-1 mRNA showed stromal and very focal epithelial expression. In all the tumor types, TAMs were most abundant at the tumor-stroma border, usually occurring singly or in small groups. The normal ovaries were all positive for stromal MCP-1 mRNA and all contained stromal macrophages. Human MCP-1 protein was detected by specific ELISA in peritoneal washouts of two different human ovarian cancer xenografts in nude mice. These tumors are associated with a significant increase in the macrophage population 7 d after injection. Although it is likely that the tumor epithelium is a significant source of MCP-1 in vivo, stromal elements also contribute. A shift in balance from stromal to epithelial MCP-1 production may be associated with a more malignant phenotype.

Although MCP-1 has been detected in other pathologies associated with a macrophage infiltrate, this is the first demonstration that it is produced by an epithelial tumor and that the tumor cells themselves are the likely major source. In human malignant glioma, MCP-1 mRNA and protein localized to tumor cells but not infiltrating macrophages (28). A similar pattern of MCP-1 expression has been demonstrated in Kaposi's sarcoma (29) and the fibroblast-like tumor cells derived from fibrous histiocytoma in culture (30). MCP-1 production by epithelial cells has been shown in other conditions associated with a macrophage infiltrate; human MCP-1 derived from vascular endothelium has been demonstrated both in animal models and human pathological specimens of atherosclerosis. The murine equivalent of MCP-1, JE, has been detected in renal tubular epithelium in a mouse model of hydronephrosis (31). The source of the stromal MCP-1 in the tumors and nonmalignant ovarian stroma that we studied is uncertain but a wide variety of other cell types are known to be able to produce MCP-1, including macrophages (32, 33), monocytes (34), and fibroblasts (35).

The mechanism by which MCP-1 acts as a chemoattractant is not clear. Martinet (36) suggests that there is an in vivo concentration gradient between the tumor and neighboring blood vessels on the basis of the presence of a monocyte chemotactic activity gradient between pleural fluid and plasma in patients with pulmonary adenocarcinoma. We also found a significant difference in MCP-1 concentration between ascitic fluid and plasma. Although there was no statistically significant correlation between the amount of MCP-1 mRNA expression and the number of infiltrating macrophages, there was a topographical association, with both the majority of MCP-1 mRNA and TAMs being found at the epithelial-stromal interface. The relationship between MCP-1 expression and TAMs is unlikely to be simple. Studies with transgenic mice show that even if MCP-1 is over expressed in the gonads, there is not an accompanying macrophage infiltrate (37). Chemokines may trigger the expression of leukocyte integrins, thereby enabling strong adhesion to vascular endothelium (38). They may also stimulate the release of matrix metalloproteases and facilitate the movement of cells through the tumor stroma (39); the matrix metalloprotease MMP-9, which degrades type IV collagen found in basement membranes, has been detected in human ovarian cancer by in situ hybridization and its distribution corresponds to that of TAMs (12).

In ovarian cancer, monocyte chemoattraction may be a function of more than one chemokine; for instance, macrophage-colony stimulating factor protein has been detected by immunohistochemistry (40). Other c-c chemokines, such as RANTES, also have monocyte chemotactic activity; furthermore, some may play a part in the recruitment of T cells, which form a prominent part of the mononuclear cell infiltrate in human ovarian cancer (2). On the other hand, MCP-1 is chemoattractant for $CD56^+/CD16^+$ natural killer cells (41) but these are rarely seen in human ovarian cancer (3). The presence of MCP-1 is therefore likely to be necessary, but not by itself sufficient, for macrophage recruitment.

We conclude that MCP-1 is expressed by tumor cells in human epithelial ovarian cancer. Furthermore, human MCP-1 is produced by ovarian cancer xenografts in nude mice and is constitutively expressed by several cell lines in vitro. In vivo, other factors, such as expression of the appropriate adhesion molecules and the correct substrate for optimal chemotaxis, are likely to be required for monocyte infiltration.

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