The CC Chemokine MCP-1/CCL2 in Pancreatic Cancer Progression: Regulation of Expression and Potential Mechanisms of Antimalignant Activity

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ABSTRACT

The aim of this study was to discover whether MCP-1/CCL2, a CC chemokine able to attract macrophages, is expressed in human pancreatic cancer and how it modulates cancer progression. All primary tumors were tested, and 6 of 14 pancreatic cancer cell lines were constitutively secreted CCL2. Analysis of the regulation demonstrated that the expression of CCL2 was significantly elevated and in a synergistic manner by IFN-γ, tumor necrosis factor α, and interleukin 1β. By immunohistochemistry and in situ hybridization, CCL2 production was confirmed in neoplastic ducts from surgical specimens. Serum levels of CCL2 in pancreatic cancer patients were significantly higher than in normal healthy subjects (P < 0.0001). Patients with high circulating levels of CCL2 had significantly higher survival rate than low CCL2 producers. Serum CCL2 levels positively correlated with tumor macrophage infiltration and inversely correlated with tumor proliferative activity (Ki67 expression). A direct effect of CCL2 on tumor cells is to be excluded, either because primary tumors as well as cell lines have no detectable CCL2 receptor (CCR2) and because addition of CCL2 on tumor cells in vitro did not modify cell cycle progression or apoptosis. In vitro, a model of tumor microenvironment showed a direct antiproliferative and proapoptotic activity of monocytes toward pancreatic cancer cell, which is mediated at least in part by interleukin 1β. Moreover, other proinflammatory cytokines such as tumor necrosis factor α and IFN-γ appeared able to induce apoptosis and to reduce the proliferative rate of pancreatic cancer. On the whole, the results presented in our investigation suggest that CCL2 could be a relevant negative regulator of pancreatic cancer progression.

INTRODUCTION

Human pancreatic cancer is a neoplasm primarily of ductal origin and is, depending on the gender, the fourth to fifth leading cause of cancer death (1). Despite pancreatic carcinoma ranking as the eighth most frequent type of solid tumor arising worldwide, it represents the fourth most frequent cause of death (2). This discrepancy reflects the current lack of effective treatment available. Despite advances in surgical as well as nonsurgical treatment efforts, it remains a tumor with poor prognosis and a 5-year survival rate of 3–8% (3–10). From these data, it appears clear that a better understanding of the fundamental nature of pancreatic cancer is needed to improve the clinical outcome. The reasons for this high mortality must be searched in both clinical and biological aspects. Insensitivity to growth inhibitory and apoptotic signals as well as self-sufficiency of growth-promoting factors are hallmarks of the pathogenesis of this malignancy (11, 12). Tumor progression is influenced by intrinsic properties of tumor cells, as well as by microenvironmental factors (13). The potential contribution of inflammation to pancreatic cancer progression motivated researchers to identify factors inducing leukocyte inflammatory recruitment at the tumor site. Potential candidates that may support such an activity are members of the chemokine superfamily (14). Several studies have focused on IL-8/CXCL8, a protein that potently induces neutrophil migration (15–22). Systematic investigation has revealed that CXCL8 is overexpressed in most human pancreatic cancer tissues and established cell lines (15, 16, 19, 23–26). Several stress factors such as hypoxia, acidosis, NO, and cell density significantly influence the expression of CXCL8 in human pancreatic cancer cells. Additionally, the expression levels correlate with its tumorigenic and metastatic potential in an orthotopic xenograft model (19, 23, 24). Multiple mechanisms are apparently involved in CXCL8 activity, including direct effects on tumor cell proliferation, angiogenesis, and indirect effects via recruitment of host-infiltrating cells such as neutrophils and macrophages, which may, in turn, produce numerous factors that promote tumor angiogenesis and growth (21). A possible role in tumor progression for another chemokine, MCP-1/CCL2, that potently induces monocyte migration was recently suggested (27, 28). The potential contribution of CCL2 to pancreatic cancer progression is of major interest because CCL2 production correlated with the levels of macrophage content in transplanted tumors in vivo (29, 30). The presence of macrophages in tumor tissues and/or their periphery has generated broad interest. Analysis of TAMs suggests that these cells have the capacity to affect diverse aspects of the immunobiology of neoplasms. Cells of the monocyte-macrophage lineage have the potential to express tumoricidal capacity and to elicit destructive reactions (31, 32). On the other hand, they have the potential to promote tumor progression by inducing vascularization, tumor growth, metastasis, stroma formation, and dissolution (33, 34). On the basis of these observations, the macrophages balance hypothesis was coined to emphasize the dual potential of TAM to influence tumor growth in opposite directions (33). The biological significance of CCL2 expression in the pancreatic cancer microenvironment appears particularly complex and has not yet been clarified. To better understand the biological significance of CCL2 in pancreatic cancer, its expression and regulation was investigated in this study. On the whole, the results presented here propose that in this human neoplasm, tumor-derived CCL2, as well as inflammatory cytokines, may play a role in determining a less aggressive behavior.
MATERIALS AND METHODS

Cell Lines. Human pancreatic carcinoma cell lines AsPC-1, Capan-1, MiaPaCa-2, Panc-1, HS 766T, A818-4, SU8686, PT45P1, PC13, HPAF II, CFPAC1, T3M4, PaCa 44, and M186 were obtained from American Type Culture Collection (Manassas, VA) or from Professor Aldo Scarpa, Department of Pathology, University of Verona (Verona, Italy), and were grown in RPMI (Biocrom, Berlin, Germany) 10% FCS (Hyclone, Logan, UT). Primary tumors from surgical specimens were enzymatically digested with collagenase-

Detection of CCL2 Expression by ELISA Assays. CCL2 and CXCL8 were detected using a sandwich ELISA as described previously (35). ELISA for CCL2 did not cross-react with closely related chemokines CCL7 and CXCL8. CCL2 secretion was measured in culture supernatants of tumors cultured as monolayers, either unstimulated or treated with IL-1β, TNF-α, and IFN-γ (all from Pepro Tech EC, London, United Kingdom) for 24 h or with anti-Fas mAb CH-11 (100 ng/ml; Kamiya Biomedical Company, Seattle, WA). Other cytokines were quantified by ELISA: IL-1β, IL-6, and TNF-α with commercial kit by Pierce Endogen (Woburn, MA).

Northern Blot Analysis and RNase Protection Assay. Total cellular RNA was prepared from pancreatic cancer cell lines. Total RNA was extracted by the guanidinium thiocyanate method, blotted, and hybridized as described previously (36). CCL2 and CCR2B cDNAs were obtained by PCR amplification of the reported sequences (37). RPA was performed using the RiboQuant MultiPrf Amplification sets (BCK-5), following the manufacturer’s instructions (PharMingen, San Diego, CA).

Real-Time PCR. Total RNA extraction from cultured cells was performed as above. cDNA was synthesized by RT-PCR using the TaqMan Reverse Transcription Reagents (Applied Biosystems). mRNA expression was analyzed by real-time PCR using Sybr Green dye and GeneAmp 5700 Sequence Detection System (PE Biosystems, Foster City, CA). The sequences of primer pairs specific for each gene (Invitrogen) were designed with Primer Express Software (Applied Biosystems). The sequences of primer pairs were as follows: iNOS: forward primer, 5'-GAGAAGGATGTCCGAGG-3'; reverse primer, 5'-AGGTCAAGAAGCTGTGAGG-3'; IL-1β forward primer, 5'-CCTACTCACTTAAAGCCCGCCT-3'; reverse primer, 5'-TTGAAACCAAAATGGCGGTCG-3'; TNF-α forward primer, 5'-ACGAATCCAACTTCCTCACA-3'; reverse primer, 5'-CCCCAATTCTCTTGGAGCAGC-3'; TGF-β forward primer, 5'-AGATTCTGTCACTGCTCCCGGA-3'; reverse primer, 5'-AAGTTGGCGATGACCCGTCG-3'; VEGF forward primer, 5'-CTCACGAGCGGAAAGACCTTG-3'; reverse primer, 5'-CTAACATCTGATTCCACGACGC-3'; MMP-9 forward primer, 5'-GCCCTGGAACCATGTGAGTAC-3'; reverse primer, 5'-CCCGATTACGTTCAACCATTTGC-3'; reverse primer, 5'-GACCTGACCAGACAGTACG-3'; reverse primer, 5'-GACTTACCACCACTTCCTACA-3'; reverse primer, 5'-AGATAGCTGACGTCG-3'; reverse primer, 5'-AGATAGCTGACGTCG-3'.

Immunohistochemistry and ISH. Immunohistochemistry for CCL2 was performed on frozen and paraffin-embedded 5-μm thick sections obtained from surgical pancreatic specimens. Serial sections were immunostained with anti-CCL2 mAbs [clones 5D3, 2A1, 9G6, and 3A3 (1 μg/ml); the clones recognize different epitopes as shown by competitive binding with peptide aa 43–65 and aa 63–85 of CCL2], CD45 (clone LCA from Dako; dilution: 1:100), CD3 (clone PS1 from Ylem; dilution: 1:50), CD68 (clone KPI from Dako; dilution: 1:100), CD15 (clone LeuM1 from Dako; dilution: 1:10), and reactivity revealed by an avidin-biotin-complex peroxidase method (Vector), using 3,3'-diaminobenzidine as chromogen. The amount of leukocytes infiltrating the tumor was evaluated as immunostained cells at ≥200 magnification (average of 10 random fields) by using a Leitz Diaplan microscope.

Proliferation activity was determined by calculating the percentage of cells exhibiting the Ki-67 antigen, a nuclear protein expressed only in cycling cells. To this aim, we used the mAb MIB-1 (Immunotech, Marseilles, France), which reacts with an epitope encoded by a 1002-bp fragment of the Ki-67 DNA, and is suitable for use in paraffin sections. MB-1 immunohistochemistry was performed on 4-μm sections mounted on glass slides treated with poly-L-lysine. Tissue sections were dewaxed, and endogenous peroxidase activity was blocked. After antigen retrieval by microwave treatment (two passages at 850 W of 5 min each), sections were incubated with nonimmune rabbit serum at room temperature for 20 min and then incubated at 37°C for 60 min with MIB-1 antibody, diluted 1:50; no MIB-1 was added to sections used as negative control samples. After rinsing, sections were washed in the dark. Slides were counterstained with hematoxylin, dehydrated, and mounted. Because MIB-1-positive cells may be unevenly distributed in a sample, the Ki67 labeling index was determined by counting a total of at least 1000 neoplastic nuclei subdivided in 10 randomly chosen fields at ×400 magnification. ISH detection of CCL2 mRNA was performed on snap frozen orchimirc carbamyl transferase sections. Briefly, 5-μm cryostat sections were fixed for 20 min in 4% paraformaldehyde, washed in buffer, dehydrated in alcohol, and then hybridized with a mixture of biotin-labeled CCL2 probe (R&D Systems, Minneapolis, MN) at a concentration of 2 ng/μl in hybridization solution at 37°C for 3 h. After stringent washes, the sections were labeled with an avidin-biotin-peroxidase complex (Vector) and 3,3'-diaminobenzidine. Negative controls were performed by omission of the probe or by using an irrelevant biotinylated probe.

Study Cohort and Follow-Up: Pancreatic Cancer Patients. Between January 1999 and October 2002, 212 patients with suspected pancreatic neoplasm were treated in two different centers: Department of Surgery San Raffaele Hospital, University VitaSalute (Milan, Italy; n = 163) and Department of Medical and Surgical Sciences, University of Padova (Padova, Italy; n = 49). The subjects were in comparable condition before admission: none of the subjects received preadmission radiation or chemotherapy. Of the 212 patients with suspected pancreatic neoplasm, the final diagnosis was: 71% ductal adenocarcinoma (n = 150); 15% others carcinoma (n = 31): peri-ampullary carcinomas; 7 pancreatic endocrine cancers; 5 carcinomas of papilla of Vater; 3 adenocarcinomas of the duodenum; 2 bile duct carcinomas; 1 undifferentiated carcinoma; 1 metastasis of kidney cancer; 7% benign neoplasm (n = 16); 7 functioning pancreatic neuroendocrine tumors; 3 serous cystadenomas; 2 solid pseudopapillary tumors; 2 benign stromal tumors of duodenum; 2 mucinous cystic neoplasms; and 7% inflammatory neoplasms (n = 15: chronic pancreatitis). Inclusion criteria for the study were histological or cytological diagnosis of ductal adenocarcinoma; no notifications of other malignancies are still available, and all slides were reviewed by an experienced pathologist (B. E. L.), who diagnosed diagnosis of ductal adenocarcinoma of the pancreas. Eighty patients with ductal adenocarcinoma were submitted to surgery with curative intent, whereas 70 were considered unrespectable. Those patients dying within 30 days after surgery were excluded by the survival analysis (surgical mortality). Survival duration was measured from the date of histological diagnosis. Follow-up of surviving patients was updated at the end of April 2003. At that point 107 patients were dead (70%), and 43 (30%) were still alive. The mean follow-up of the survivors was 476 ± 236 days.

All of the clinical characteristics of patients were compared with the χ² test for categorical variables. Student’s t test or Anova test for unpaired data were used to compare mean values. Mann-Whitney U or Kruskal-Wallis H test were used to compare data showing non-Gaussian distribution. The effect of CCL2 was assessed by univariate analysis by computing the Hazard ratio and 95% CI presented. All probability values were two-sided. Analyses were performed using the SPSS Statistical Analysis Software. Values are presented as averages ± SD unless differently stated. Apoptosis and Cell Cycle Analysis. Human pancreatic cancer cell lines were seeded at 5 × 10⁵ cells/ml in a volume of 1.5 ml in 12-well Costar plates (Corning, Inc.). Cells were treated in duplicate with 10 ng/ml TNF-α, 10 ng/ml IL-1β, 1000 units/ml IFN-γ, 10 μM 5-fluouracil, 1 mM gemcitabine. After

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72 h, cells were trypsinized, washed once with PBS, and fixed with 70% ethanol at −20°C for 24 h. Fixed cells were washed three times and incubated for 1 h with a PI (Sigma Chemical Co.) solution (20 μg/ml) containing 0.1 mg/ml RNase A (Sigma Chemical Co.). Cells were then subjected to cell cycle analysis for determining DNA contents by flow cytometry. Cell debris was excluded on the basis of forward versus side scatter. Doublets and clumps were excluded by gating on a bivariate distribution of AUX (PI peak pulse) versus the PI integrated signal. Data from 10,000 events were collected in the final gated histograms.

**Monocytes and Tumor Cell Coculture.** Peripheral blood monocytes were obtained by density gradients and plastic adhesion as described previously (38). To evaluate the effects of monocyte on pancreatic cancer cells, monocytes (10^6/ml) were seeded in the upper chamber of 6-well Transwell plates with permeable membrane pores (0.2 μm; Costar Corning Inc., Corning, NY). Tumor cell lines (1 × 10^5) were seeded in the lower chamber. At day 3, cancer cells were harvested and used in apoptosis and cell cycle analysis. Neutralizing Abs were used at 10 μg/ml and included anti-IL-1β and anti-TNF-α (R&D Systems).

**RESULTS**

**Detection of CCL2 Expression by Pancreatic Cancer Cells in Vitro: Regulation by Proinflammatory Cytokines.** We systematically determined CCL2 expression in 14 human pancreatic cancer cell lines under in vitro culture conditions. Six of 14 cell lines (40%) constitutively expressed high levels of CCL2 in vitro as measured using ELISAs (Fig. 1A). Northern blot analysis confirmed the constitutive expression of CCL2 by the pancreatic cancer cell lines (Fig. 1A). The same blot was hybridized for CCR2 (receptor of CCL2). CCR2 mRNA was undetectable in all of the cell lines (Fig. 1A). To study whether other chemokines are secreted by tumor cells, we performed an RPA from 2 different cell lines (AsPC-1 and Panc-1; Fig. 1B). No mRNA for I-309/CCL1, MIP-1α/CCL3, MIP-1b/CCL4, IFN-inducible protein 10/CXCL10, IL-8/CXCL8, LPTN/XCL1, or RANTES/CCL5 was detected in resting conditions in these 2 cell lines. CXCL8 was measured using ELISAs in all of the cell lines. Eight of 14 cell lines (PT45P1, MiaPaca2, Capan-1, CFPAC1, SU8686, T3M4, HS766T, and HPAF II) constitutively expressed high levels of CCL2 in vitro and did not correlate with CCL2 secretion (data not shown).

To evaluate the possibility that cytokines produced by tumor cells or by adjacent stromal cells and/or inflammatory cells may regulate CCL2 expression, we determined the ability of IFN-γ, TNF-α, IL-1β, IL-10, IL-6, and HGF to affect CCL2 secretion by 13 pancreatic cancer cell lines. As shown in Table 1, CCL2 secretion was up-regulated by TNF-α, IL-1β, and IFN-γ in constitutively expressing cell lines and was induced at low levels in 4 of 8 of negative cell lines. On the other hand, IL-10, IL-6, and HGF did not substantially modify CCL2 secretion. Up-regulation of CCL2 by inflammatory cytokines was confirmed by Northern blot analysis (Fig. 1C). The same blot also shows that CCR2 was not expressed even after cytokines treatment. Interestingly, TNF-α, IL-1β, and IFN-γ synergized in their ability to promote CCL2 expression. Because it was recently proposed that Fas stimulation could induce chemokine secretion in pancreatic cancer (28), suggesting that Fas stimulation could contribute to the accumulation of immune cells at the tumor site, we investigated the production of CCL2 after treatment with anti-Fas mAb in 7 human pancreatic cancer cell lines. Ligation of Fas on tumor cells did not induce CCL2 secretion in nonproducing cell lines and did not increase release in constitutively expressing cell lines (data not shown).

**In Vivo Expression of CCL2 in Pancreatic Cancer: Correlation with TAM Infiltration and Proliferative Activity.** With the objective to investigate the in vivo role of CCL2 in the natural history of pancreatic cancer, serum concentrations of CCL2 in 212 patients with suspected pancreatic neoplasm and in 159 healthy subjects were determined. CCL2 median level in the healthy group was 67 pg/ml (25th–75th percentile: 62–74 pg/ml). CCL2 median level in patients with final diagnosis of pancreatic cancer was significantly higher: 105 pg/ml in ducal adenocarcinoma (80–133, P = 0.0001, n = 150); and 90 pg/ml in other carcinomas (70–126 pg/ml, P = 0.0001, n = 31). On the other hand, in patients with final diagnosis of inflammatory nonneoplastic disease (n = 15) or benign neoplasm of pancreas (n = 16), median levels of serum CCL2 were not significantly
different from the healthy group (80 pg/ml, 62.5–100, P = 0.06; 70 pg/ml, 50.9–87.5, P = 0.35 respectively; Fig. 2A).

CCL2 expression was analyzed in sections of pancreatic surgical specimens from 10 patients. All of the specimens tested stained positive for CCL2 by immunohistochemistry (Fig. 3A) and ISH (Fig. 3B). Primary cultures of pancreatic cancer cells from surgical specimens were also tested from 4 patients. Tumor cells grew as adherent monolayers and were positive for CCL2 by immunohistochemistry (Fig. 3B). In these 4 patients, CCL2 levels remained stable in N-group (from 80 to 99 pg/ml; 91 to 120 pg/ml, respectively; P = 0.035; n = 16), increased in L-group (from 80 ± 2 to 80 ± 4 pg/ml; P = 0.64; n = 9), and increased in L-group (from 40 ± 6 to 76 ± 9 pg/ml; P = 0.01).

We divided patients with a final diagnosis of ductal adenocarcinoma (n = 150) in three groups according to CCL2 serum levels: low group (L-group, n = 27) with CCL2 values below the 10th percentile (57 pg/ml) of healthy subjects; normal group (N-group, n = 47) with CCL2 values between the 10th and 90th percentiles; and high group (H-group, n = 76) with CCL2 values above the 90th percentile (91 pg/ml). The clinical characteristics of these groups are summarized in Table 2. No differences in terms of age, sex, size, tumor localization, presence of metastasis and vessel infiltration, resectability, and nonsurgical treatment were found. To prove that the source of systemic CCL2 was the tumor microenvironment, we evaluated if ablative surgery could affect the levels of circulating CCL2. Serum concentrations were measured in 36 patients with ductal adenocarcinoma of the pancreas before surgery and 14 days after operation (Fig. 2B). After radical surgery, CCL2 levels significantly dropped in H-group (from 145 ± 91 to 120 ± 27 pg/ml; P = 0.035; n = 16), remained stable in N-group (from 80 ± 2 to 80 ± 4 pg/ml; P = 0.64; n = 9), and increased in L-group (from 40 ± 6 to 76 ± 9 pg/ml; P = 0.01).

**Table 1** Secretion of CCL2 protein from human pancreatic cancer cells after stimulation with IL-1β (10 ng/ml), TNF-α (10 ng/ml), IFN-γ (1000 units), IL-10 (10 ng/ml), IL-6 (10 ng/ml), and HGF (10 ng/ml), or medium alone for 24 h.

<table>
<thead>
<tr>
<th>Line</th>
<th>Ccl2 (pg/ml/24 h)</th>
<th>IL-1β</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>TNF-α+IL-1β</th>
<th>TNF-α+IL-1β</th>
<th>IL-10</th>
<th>IL-6</th>
<th>HGF</th>
</tr>
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<tr>
<td>Ctrl</td>
<td>0.3 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Ccl2</td>
<td>0.3 ± 0.3</td>
<td></td>
<td></td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>AsPC1</td>
<td>0 ± 0</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MiaPac2</td>
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<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T3M4</td>
<td>0 ± 0</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Pacc4</td>
<td>0 ± 0</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>HPAF II</td>
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<td></td>
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<td>A8184</td>
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<tr>
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<tr>
<td>M186</td>
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**A. Constitutively expressing CCL2**

**B. Not constitutively expressing CCL2**

**Fig. 2.** Serum CCL2 in pancreatic diseases. A, serum CCL2 levels in healthy subjects (Lane 1, ctrl), patients with malignant pancreatic tumors (Lane 2, ductal carcinomas; Lane 3, other carcinoma), benign pancreatic tumors (Lane 4), and pancreatic inflammatory diseases (Lane 5). The gray zone represents the range between 10th and 90th percentiles of CCL2 levels in ctrl population. Horizontal lines: median and 25th-75th percentiles. The right table reports the distribution of patient by final diagnosis. The median values and the 10th–90th percentiles of CCL2 serum levels in the different groups are reported. B, serum CCL2 concentration was measured in 36 patients with ductal pancreatic cancer 1 day before ablative surgery (presurgical CCL2) and 14 days after surgery (postsurgical CCL2). C, serum CCL2 concentration was measured simultaneously in peripheral vein and in portal vein during the surgery in 53 patients with ductal pancreatic adenocarcinoma.
P = 0.02; n = 11). To demonstrate a CCL2 gradient between tumor draining blood and peripheral blood, samples were simultaneously collected from a peripheral vein and the portal vein during surgical resection in 53 patients (Fig. 2C). In H-group, the CCL2 concentration was significantly higher in portal vein than in peripheral vein (170 ± 10 and 140 ± 9 pg/ml, respectively, P = 0.001; n = 17). Similar results were obtained in N-group even if with a lower peripheral/portal vein gradient (110 ± 10 and 90 ± 9 pg/ml, P = 0.001; n = 17), whereas in L-group, no significant difference was observed (56 ± 12 versus 52 ± 5 pg/ml, P = 0.4; n = 19). These results strongly indicate that in those patients with high levels of CCL2, chemokine secretion derives from the tumor site.

The clinicopathological factors (pathological tumor-node-metastasis, grading, anatomical size, percentage of MIB-1-positive cells; n = 80) and the leukocyte infiltration (n = 20) were evaluated in patients undergoing surgery (Table 3). CCL2 serum levels did not significantly correlate with pathological tumor-node-metastasis, histological grade, or anatomical size. A statistically significant difference was found in terms of macrophage infiltration between the three groups (percentage of macrophage 24 ± 4; 46 ± 8; 57 ± 12 for L-, N-, and H-group, respectively; P = 0.05). These results suggest also for pancreatic cancer, a role of CCL2 in macrophage recruitment in vivo. The proliferative index of cancer appeared influenced by CCL2. In fact, the percentage of Ki-67-positive cells decreased with the increase of CCL2 (percentage of Ki-67-positive cells: 34 ± 6; 30 ± 3; 21 ± 2 for L-, N-, and H-group, respectively; P = 0.04).

Univariate and Multivariate Analysis: CCL2 Secretion Is an Independent Favorable Prognostic Factor in Resected Pancreatic Cancer Patients. To understand if CCL2 secretion by pancreatic cancer may play a role as prognostic marker regarding overall survival, we correlated CCL2 serum levels with the survival time of our population (n = 150). Patients undergoing surgery (n = 80) were considered separately from nonsurgical patients (n = 62). The reason why surgical and nonsurgical patients with pancreatic cancer were analyzed separately is because patients not undergoing surgical resection have in general a very bad prognosis because of the presence of distant metastasis or local advance disease (great vessel invasion) and poor general conditions. For this analysis, the L- and N-groups were merged in one group (L-/N-group, n = 74). Eight patients were excluded by the analysis for the absence of information on survival. In patients treated with surgical resection, univariate analysis showed that positive margins of resection (R1), presence of metastasis (pM1), low grading (G1–2), and high serum levels of CCL2 (H-group) are strongly associated with survival (Table 4). The multivariate analysis

### Table 2. CCL2 secretion by pancreatic cancer in vivo: clinical characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal (ng/ml)</th>
<th>Δ Pre/post</th>
<th>Δ Per/portal</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>CA 19.9</th>
<th>Creatinine</th>
<th>Size (cm)</th>
<th>Localization</th>
<th>Vessel invasion</th>
<th>Metastasis</th>
<th>CT/RT</th>
<th>Surgical treatment</th>
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<tr>
<td>Low</td>
<td>54 ± 12</td>
<td>1 ± 8</td>
<td>65 ± 11</td>
<td>12/15</td>
<td>1.05 ± 0.9</td>
<td>3.2 ± 1</td>
<td>12/15</td>
<td>74%</td>
<td>52%</td>
<td>52%</td>
<td>61%</td>
<td>61%</td>
<td>74%</td>
</tr>
<tr>
<td>Normal</td>
<td>80 ± 15</td>
<td>5 ± 5</td>
<td>66 ± 8</td>
<td>28/19</td>
<td>0.87 ± 0.23</td>
<td>3.9 ± 1</td>
<td>82%</td>
<td>82%</td>
<td>54%</td>
<td>54%</td>
<td>71%</td>
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<td>53%</td>
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<tr>
<td>High</td>
<td>130 ± 21</td>
<td>24 ± 76</td>
<td>67 ± 1</td>
<td>42/34</td>
<td>0.9 ± 0.31</td>
<td>5.2 ± 2</td>
<td>80%</td>
<td>51%</td>
<td>27%</td>
<td>27%</td>
<td>61%</td>
<td>61%</td>
<td>46%</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0002</td>
<td>0.0164</td>
<td>0.7</td>
<td>0.8</td>
<td>0.69</td>
<td>0.29</td>
<td>0.39</td>
<td>0.69</td>
<td>0.94</td>
<td>0.64</td>
<td>0.46</td>
<td>0.06</td>
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</table>

**Fig. 3.** Expression of CCL2 in human pancreatic cancer cell in vivo. A and B, immunohistochemical localization of CCL2 and ISH in human pancreatic cancer (left) and normal pancreatic tissue (right). A, representative examples (×200, ×400) of paraffin sections stained with antibodies to CCL2 (SD3-F7). Specificity for CCL2 was confirmed with three other mAbs able to recognize different epitopes of CCL2; all of the antibodies showed the same pattern of positivity (data not shown). B, by ISH, CCL2 mRNA is detected in ductal cells from human pancreatic cancer cell but not in normal ductal cell. C, CCL2 expression was determined at the protein level in supernatants of 4 human pancreatic tumor cell primary cultures by ELISA. D, serum CCL2 concentrations in peripheral vein and in portal vein in patients whose tumor primary cultures are shown in C.
was performed using variables significant at \( P < 0.2 \) in univariate analysis (tumor localization and adjuvant treatments were also added for their clinical relevance) and confirmed the relevant role of high CCL2 concentration as favorable independent prognostic factor in resected patients \{Cox proportional hazard models of the predictors \( \text{mortality by multivariate analysis: hazard ratio (95\% CI)} \}

In patients not treated with surgical resection, RT/CT and the presence of metastasis and vessel invasion appeared the more relevant factors determining survival (Table 4). In these patients, the survival rate was not different in normal/low \( (n = 24) \) and high \( (n = 38) \) CCL2 group (median survival, respectively, 350 + 82 versus 681 + 126 days; \( P = 0.043 \); Fig. 4A).

**Direct and Indirect Effects of CCL2 on Pancreatic Tumor Cells.** A mechanism by which chemokines may affect tumor progression is by acting directly on the proliferation of tumor cells via an autocrine loop. This mechanism, in pancreatic cancer, was previously described for IL-8/CXCL8 \( (15, 18) \). A direct effect of CCL2 on pancreatic tumor cells would require the expression of the specific receptor CCR2. As shown in Fig. 1, A–C, mRNA for CCR2 was undetectable in all of the tumor cell lines and was not up-regulated by inflammatory cytokines. FACS analysis with anti-CCR2 mAb revealed no CCR2 expression (data not shown). We also analyzed the expression of CCR2 in four primary cultures of pancreatic cancer by RT-PCR and in sections of pancreatic carcinoma by immunohistochemistry. All of the primary cultures and the pancreatic cancer sections tested were negative for CCR2 (data not shown). Although no expression of CCR2 mRNA and protein were detected in pancreatic cancer, it is not possible to rule out the presence of other still unidentified CCL2 receptor(s) able to transduce signals from CCL2. Therefore, we evaluated the proliferation rate and the modulation of apoptosis in pancreatic cancer cell lines after exposure to CCL2. In a range of concentrations from 10 ng/ml to 1 \( \mu \)g/ml, CCL2 did not induce significant modification of the cell cycle or cell death as shown by DNA content analysis (Fig. 5) and CSFE analysis by flow cytometry (data not shown). On the whole, these results exclude a direct effect of CCL2 in pancreatic tumor cell lines.

A second mechanism by which chemokines may indirectly affect tumor progression is by acting on stromal cells. CCL2 production correlates with the levels of tumor-associated macrophages content in tumors \( in vivo \) \( (30, 39) \) as confirmed also in this study. Monocyte/macrophages play a dual role in neoplasm: they may kill neoplastic cells after activation or promote neoplastic progression by secreting growth factors. To evaluate the specific effects of monocyte/macrophages on pancreatic cancer cells, monocytes were seeded with or without CCL2 (100 ng/ml) in the upper chamber of Transwell plates

### Table 3 CCL2 secretion by pancreatic cancer in vivo: clinicopathological characteristic

<table>
<thead>
<tr>
<th>n</th>
<th>pT (%)</th>
<th>pN (%)</th>
<th>pM (%)</th>
<th>G (%)</th>
<th>Proliferation (MIB)</th>
<th>No. total leukocytes</th>
<th>Macrophage (CD68+)</th>
<th>Polymorphonuclear (CD15+)</th>
<th>T Ly (CD3+)</th>
<th>DC (CD1a+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>20</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>35</td>
<td>65</td>
<td>79</td>
<td>21</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>Normal</td>
<td>25</td>
<td>0</td>
<td>84</td>
<td>16</td>
<td>12</td>
<td>88</td>
<td>88</td>
<td>12</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>High</td>
<td>35</td>
<td>10</td>
<td>62</td>
<td>28</td>
<td>28</td>
<td>78</td>
<td>91</td>
<td>9</td>
<td>12</td>
<td>50</td>
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### Table 4 Cox proportional hazard models of the predictors of pancreatic cancer mortality by univariate and multivariate analysis in the population divided by surgical resection

**A. Univariate analysis**

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR (95% CI)</th>
<th>( P )</th>
<th>HR (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resected patient</td>
<td>Unresected patient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (M)</td>
<td>0.90 (0.51–1.59)</td>
<td>0.72</td>
<td>1.08 (0.62–1.87)</td>
<td>0.78</td>
</tr>
<tr>
<td>Age</td>
<td>1.01 (0.97–1.03)</td>
<td>0.74</td>
<td>1.02 (0.98–1.05)</td>
<td>0.23</td>
</tr>
<tr>
<td>CCL2 (high)</td>
<td>0.51 (0.26–0.99)</td>
<td>0.05</td>
<td>0.90 (0.50–1.61)</td>
<td>0.734</td>
</tr>
<tr>
<td>RT/CT</td>
<td>0.72 (0.38–1.36)</td>
<td>0.32</td>
<td>0.36 (0.19–0.66)</td>
<td>0.001</td>
</tr>
<tr>
<td>Vessel invasion</td>
<td>0.82 (0.45–1.5)</td>
<td>0.52</td>
<td>0.44 (0.24–0.80)</td>
<td>0.008</td>
</tr>
<tr>
<td>Size</td>
<td>1.04 (0.79–1.37)</td>
<td>0.74</td>
<td>0.97 (0.72–1.29)</td>
<td>0.82</td>
</tr>
<tr>
<td>Localization (head)</td>
<td>0.74 (0.35–1.57)</td>
<td>0.44</td>
<td>0.87 (0.46–1.63)</td>
<td>0.66</td>
</tr>
<tr>
<td>Low pN (2)</td>
<td>0.96 (0.50–1.8)</td>
<td>0.90</td>
<td>0.55 (0.23–1.31)</td>
<td>0.18</td>
</tr>
<tr>
<td>Negative lymphnodes (pN0)</td>
<td>2.24 (1.03–4.29)</td>
<td>0.04</td>
<td>2.09 (1.18–3.69)</td>
<td>0.012</td>
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<tr>
<td>Metastasis (pM1)</td>
<td>0.48 (0.26–0.88)</td>
<td>0.019</td>
<td>0.94 (1.01–3.74)</td>
<td>0.046</td>
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<tr>
<td>Low grading (G1–2)</td>
<td>1.01 (0.97–1.04)</td>
<td>0.52</td>
<td>1.00 (1.00–1.00)</td>
<td>0.07</td>
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<tr>
<td>MIB</td>
<td>1.94 (1.01–3.74)</td>
<td>0.046</td>
<td>1.00 (1.00–1.00)</td>
<td>0.55</td>
</tr>
<tr>
<td>CA19.9</td>
<td>1.00 (1.00–1.00)</td>
<td>0.07</td>
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</table>

**B. Multivariate analysis**

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<th>Variables</th>
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<th>( P )</th>
<th>HR (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resected patient</td>
<td>Unresected patient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL2 (high)</td>
<td>0.42 (0.18–0.95)</td>
<td>0.037</td>
<td>1.23 (0.55–2.73)</td>
<td>0.60</td>
</tr>
<tr>
<td>RT/CT</td>
<td>0.52 (0.24–1.09)</td>
<td>0.085</td>
<td>0.43 (0.21–0.88)</td>
<td>0.02</td>
</tr>
<tr>
<td>Vessel invasion</td>
<td>0.48 (0.15–1.46)</td>
<td>0.19</td>
<td>0.45 (0.17–1.16)</td>
<td>0.09</td>
</tr>
<tr>
<td>Negative lymphnodes (pN0)</td>
<td>1.91 (0.77–4.7)</td>
<td>0.16</td>
<td>1.59 (0.75–3.36)</td>
<td>0.22</td>
</tr>
<tr>
<td>Metastasis (pM1)</td>
<td>0.46 (0.23–0.91)</td>
<td>0.026</td>
<td>1.00 (1.00–1.00)</td>
<td>0.98</td>
</tr>
<tr>
<td>Low grading (G1–2)</td>
<td>1.12 (0.52–2.4)</td>
<td>0.75</td>
<td>1.00 (1.00–1.00)</td>
<td>0.07</td>
</tr>
</tbody>
</table>
with permeable membrane pores (0.2 µm). Six different tumor cell lines were seeded in the lower chamber: Panc-1; PT45Pl; Capan-1; Hs766T (CCL2-producer cell lines); and MiaPaCa 2 and AsPC 1 (non-CCL2-producers). After 72 h, culture tumor cells were harvested, and proliferation and apoptosis were evaluated in flow cytometry for DNA content analysis and confirmed by optical microscopy. After exposure to monocytes, the percentage of apoptotic cells (sub G1 peak) increased significantly compared with ctrl (tumor cells alone) cultures (Fig. 5). Monocytes induced apoptosis both in nonproducer cell lines, as well as in 3 of 4 CCL2-producing cell lines. Addition of CCL2 to monocyte/tumor cocultures resulted in higher apoptosis, but this increase was less pronounced in CCL2-secreting tumor cells (Fig. 5).

The DNA content analysis also showed that the percentage of tumor cell in G2 phase and concordantly the G2-G1 ratio were decreased after coculture with monocytes.

To evaluate if the monocyte-induced effects could be mediated by inflammatory cytokines secreted by monocytes, blocking anti-IL-1β and anti-TNF-α mAbs were added. As shown in Fig. 6, anti-IL-1β but not anti-TNF-α reverted the monocyte-induced effects, indicating that IL-1β was the most relevant factor that induced apoptosis and cell cycle modification in this coculture system. To confirm that inflammatory cytokines affected the cell cycle and survival of pancreatic cancer cells, three different pancreatic cancer cell lines (Capan 1, Hs766T, and MiaPaCa2) were studied after exposure to IL-1β (10 ng/ml), TNF-α (10 ng/ml), and IFN-γ (1000 units/ml). As shown in Fig. 7, all of the three cytokines were able to induce apoptosis in pancreatic cancer cell lines. The mean fold increase versus untreated cells was 2.31 ± 0.3, 2.1 ± 0.2, and 1.82 ± 0.25 for IL-1β (P = 0.02) and TNF-α (P = 0.02) and IFN-γ (P = 0.04), respectively (Fig. 7). Combination of IL-1β and TNF-α or TNF-α and IFN-γ had a synergistic effect in inducing apoptosis. The cell cycle was also influenced by cytokines. The G2-G1 ratio decreased from 1.037 ± 0.3 to 0.516 ± 0.06 (P = 0.01) and 0.616 ± 0.09 (P = 0.02) after exposure to IL-1β/TNF-α and TNF-α/IFN-γ, respectively (Fig. 7C).

CCL2 but also other Tumor-Derived Factor(s) Cooperate in Monocyte Activation. To verify whether CCL2 had a direct effect on inflammatory cytokine production by monocytes, we investigated the expression of IL-1β, TNF-α, and iNOS after CCL2 stimulation (Fig. 8A) shows that resting monocytes exposed to CCL2 in vitro for 6 h expressed considerable higher amounts of iNOS compared with untreated and even LPS-treated monocytes. IL-1β and TNF-α were not up-regulated by CCL2. iNOS generates NO, a molecule known to induce G1 arrest followed by apoptosis in pancreatic tumor cell lines (40). Thus, the production of iNOS by CCL-2-treated monocytes could explain the increased apoptosis of tumor cells after addition of CCL2 in cocultures experiments. To further investigate whether CCL2 modulated also the expression of other monocyte-derived factors that have an impact on tumor growth and dissemination, we evaluated the expression of TGF-β, VEGF, and MMP-9 in CCL2-treated monocytes. None of these molecules was significantly modulated by CCL2 (Fig. 8B).

The fact that monocytes were able to induce apoptosis also in CCL2-nonproducing cell lines indicated that factors different from CCL2 could be released by tumor cells and could stimulate the production of inflammatory cytokines by monocytes. To address this question, monocytes were cocultured in Transwell plates (0.2 µm pores) for 3 days with CCL2-producing and CCL2-nonproducing tumor cells. At the end of culture, cytokines were measured by ELISA in the supernatants. IL-1β and IL-6, but not TNF-α, were present in the coculture supernatants with both CCL2+ and CCL2− cell lines, although slightly higher with CCL2 + tumor cells (Fig. 8B). To investigate the cellular source of cytokines, we separately tested
mRNA from tumor cells and monocytes by RT-PCR. All of the pancreatic tumor cell lines were negative for IL-1β/9252 (data not shown), whereas cocultured monocytes expressed 7–15-fold more IL-1β compared with untreated monocytes (100-fold with LPS-treated monocytes used as reference stimulus), confirming that the source of the inflammatory cytokines are indeed the monocytes. Overall, these results indicate that monocytes cocultured with pancreatic tumor cells release inflammatory cytokines (e.g., IL-1), which promote tumor apoptosis. CCL2 is not indispensable, but its presence favors tumor death by inducing other cytotoxic factors (e.g., iNOS).

DISCUSSION

In this study, we demonstrate that the chemokine CCL2 is expressed and secreted in vitro and in vivo by pancreatic tumor cells and that inflammatory cytokines up-regulated its expression. CCL2 is probably the CC chemokine most frequently found in tumors because of its description as a tumor-derived chemotactic factor (29). Human tumors shown to express CCL2 in vivo include sarcomas, gliomas, and lung tumors, carcinomas of the breast, cervix, and ovary, and melanoma (41). Several investigations have provided recent evidence for the potential contribution of monocyte chemoattractants to cancer progression (42–45). However, the significance of tumor infiltration by host cells and the precise role of chemokines as regulators of leukocyte recruitment in malignant tumors have not been completely clarified. In this respect, our study sheds some light on the regulation of CCL2 expression in pancreatic cancer and on the potential mechanisms by which it may contribute to cancer growth. Our results with tumor cell lines producing CCL2 confirms and extends previous recent findings on 3 pancreatic cancer cell lines (27, 28). Interestingly, of 14 cell lines tested, only 6 constitutively produced detectable levels of CCL2. Of these, 4 cell lines showed a statistically significant increase in CCL2 expression upon treatment with inflammatory cytokines (27, 28). The remaining cell lines were not fully analyzed for CCL2 expression, but their production of other CC chemokines, such as CCL3 and CCL4, was not detectable (27, 28). These findings suggest that CCL2 may play a role in the regulation of leukocyte recruitment in pancreatic tumor microenvironments.

**Fig. 6.** IL-1β is the soluble mediator of monocyte-induced apoptosis in pancreatic cancer cell. Coclultures of pancreatic cancer cells and monocytes were incubated for 72 h in the presence or absence of blocking antibodies against IL-1β, TNF-α, or both. Anti-IL-1β but not anti-TNF-α reverted the monocyte-induced effects on apoptosis and cell cycle. DNA content is demonstrated by PI staining after permeabilization of cells and analyzed by FACS. Numbers are percentage of the events analyzed. Representative of four experiments.

**Fig. 7.** Inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) synergize in inducing apoptosis and cell cycle modification in pancreatic cancer cells. Cell cycle and apoptosis were evaluated in 6 different pancreatic cancer cell lines (Capan, Panc1, Hs766T, AsPC-1, MiaPac2, and Paca44) after exposure to IL-1β (10 ng/ml), TNF-α (10 ng/ml), and IFN-γ (1000 units/ml) for 72 h. A, representative profile from AsPC-1 and Capan-1 cell lines; percentage of cells in apoptosis (sub-G1), G0/G1 phases (G1 peak), and S-G2 phases (G2 peak) of the cell cycle. DNA content was evaluated by propidium iodide staining after permeabilization of cells and analyzed by FACS. Representative of four experiments. B, apoptosis fold increase of apoptosis respect ctrl. Values are the mean of the 6 cell lines examined. *, P < 0.05; **, P < 0.01. C, G2/G1 ratio. Values are the mean of the 6 cell lines examined. *, P < 0.05; **, P < 0.01.
tumor release inflammatory cytokines, including TNF-α. Pancreatic tumor cells could promote the recruitment of monocyte/macrophages, assessed by immunohistochemistry. This finding is in line with previous reports on different tumors (29, 39). One can envisage a cytokine network at the tumor site. CCL2 produced by tumor cells was confirmed, although in a small number of patients, that patients with high serum level of CCL2 had higher amounts of tumor-infiltrating macrophages. Macrophages have complex dual functions in their interaction with neoplastic cell. Whereas macrophages of M1 phenotype have the potential to kill neoplastic cells after activation by IL-2 and IFNs (32, 49), M2 macrophages produce a number of potent angiogenic and lymphangiogenic growth factors, cytokines, and proteases, all mediators that potentiate neoplastic progression (50). Moreover, chemokines are potent activators of proteases that facilitate macrophage-mediated digestion of extracellular matrix (51, 52). In particular, CCL2 potently activates gelatinase, uPA, and its receptor (51, 53). The tissue-type plasminogen activator, uPAs, and their respective receptors, annexin II and u-PAR, were demonstrated to contribute to the invasive behavior of pancreatic cancer (54, 55). MMP-2 expression appeared to be an important determinant of pancreatic cancer dissemination as levels of active MMP-2 strongly correlates with nodal status and tumor stage (56). Thus, CCL2 may indirectly contribute to pancreatic cancer dissemination by favoring the leukocyte-mediated digestion of extracellular matrix. The evidence that CCL2 was not associated with metastasis, lymph node involvement, vessel invasion, and histological differentiation of the tumor suggested that this chemokine is not relevant for the dissemination of pancreatic tumor. Others have proposed a dual role of CCL2 on tumor growth. CCL2 can be protective in some tumor models but destructive in others: murine colon carcinoma cells expressing CCL2 fail to metastasize when injected into mice (57), whereas other carcinoma cells show enhanced metastasis (58). Overexpression of CCL2 by tumor cells can lead to their destruction by an infiltrate of activated mononuclear cells (59–62). Moreover, even if many clinical studies suggest that TAM are a part of inflammatory circuits that promote tumor progression (31, 34), other clinical evidences indicated that inflammation and chemokines may also impair tumor growth and metastasis (63). A recent careful analysis of the impact of CCL2 on tumor growth in a nontumorigenic melanoma system revealed a biphasic effect (64). Low-level CCL2 secretion, with physiological accumulation of TAM, promoted tumor formation, whereas high CCL2 secretion resulted in massive macrophage infiltration into the tumor mass and its destruction.

Our in vitro data that CCL2 production by tumor cells is upregulated by treatment with IL-1β, TNF-α, and IFN-γ suggest that tumors producing in vivo high levels of CCL2 may reside in a microenvironment characterized by the presence of inflammatory cytokines. M1 macrophages rather than M2 macrophages (65) produce inflammatory cytokines, and we demonstrate in this study that the proapoptotic effect induced by cocultured monocytes is, at least in part, mediated by IL-1β. Moreover, we show that inflammatory cytokines are directly cytotoxic for pancreatic tumor cells and decrease their G2-M ratio. The mechanisms by which proinflammatory

---

**A**

Fig. 8. CCL2 but also others tumor-derived factor(s) cooperates in monocytes activation. A, resting monocytes were stimulated for 6 h with CCL2 (100 ng/ml) or LPS (10 ng/ml). Expression of IL-1β, TNF-α, iNOS, VEGF, TGF-β, and MMP-9 were performed by RT-PCR. Experiments were performed in triplicate for each sample. For each gene, mRNA was normalized (ΔΔCt) to GAPDH mRNA by subtracting the cycle threshold (Ct) value of GAPDH mRNA from the Ct value of the gene. Fold difference compared with control (2−ΔΔCt) was calculated. This is representative of at least three experiments. B, CCL2-positive (CCL2+/H9251) and CCL2-negative (CCL2−/H9004) cell lines were cultured in a Transwell system for 3 days with monocytes or activated with LPS. IL-1β, TNF-α, and IL-6 were assayed by ELISA. This is representative of at least three experiments.

---

**B**

of CCL2, whereas 4 of 4 primary tumor cultures tested in ELISA and all of the section tested in immunohistochemistry produced CCL2. This discrepancy between established tumor cell lines and primary tumors suggests that the ability to produce CCL2 has been lost during culture selection.

Our study first demonstrates that CCL2 is released in the circulation in pancreatic cancer patients very likely from the tumor microenvironment. This is supported by the higher concentration of the chemokine in the tumor draining blood, as well as by the lower CCL2 serum levels after surgical removal of the tumor. Patients with high circulating CCL2 have higher numbers of CD68+ tumor-infiltrating macrophages, assessed by immunohistochemistry. This finding is in line with previous reports on different tumors (29, 39). One can envisage a cytokine network at the tumor site. CCL2 produced by pancreatic tumor cells could promote the recruitment of monocyte/macrophages. Immune cells or other stromal cells (17, 46) within the tumor release inflammatory cytokines, including TNF-α, IL-1β, and IFN-γ, which, in turn, increase CCL2 production and amplify monocyte recruitment.

In this study, we found that circulating levels of CCL2 inversely correlated with Ki-67 Ag, a well-established marker of tumor cell proliferation. Moreover, patients undergoing surgical resection with high CCL2 serum level have a significantly higher survival rate compared with low producer patients. Thus, high CCL2 is associated with patients with a more favorable prognosis. In contrast, serum levels of CCL2 did not correlate with improved survival in patients not undergoing surgical resection. This is not an unexpected finding and reflects at least, in part, the peculiar aspects of unresectable patients. In fact, patients not eligible for surgery include patients with distant metastasis (systemic advanced disease), patients with large vessels infiltration (locally advanced disease), patients with poor general conditions or concomitant disease (i.e., cardiovascular), which all have a very poor prognosis. The overall median survival for unresected patients in this study was 188 ± 21 days, consistent with results reported by other groups (47, 48). Obviously in these compromised patients the advanced disease, together with cachexia and anorexia, play a more relevant role on survival than intrinsic properties of the tumor.

We asked whether CCL2 could directly affect pancreatic tumor cell proliferation by an autocrine loop, as previously described for CXCL8 in this tumor type (15, 18). A direct effect of CCL2 on tumor cells would require the expression of specific receptors. The absence of CCR2 mRNA and protein in tumor cell line and in primary tumors excluded that CCL2 could affect tumor cells through its canonic receptor. Potential contribution of CCL2 on tumor cell proliferation/apoptosis was tested and gave negative results. Thus, the likely conclusion is that CCL2 is not directly affecting pancreatic tumor cells, at least as far as in vitro cell cycle analysis of tumor cell lines is concerned.

CCL2 is chemotactic for monocytes and its expression in tumor has been correlated with macrophage infiltration (29, 30). In our study, we confirmed, although in a small number of patients, that patients with a high serum level of CCL2 had higher amounts of tumor-infiltrating macrophages. Macrophages have complex dual functions in their interaction with neoplastic cell. Whereas macrophages of M1 phenotype have the potential to kill neoplastic cells after activation by IL-2 and IFNs (32, 49), M2 macrophages produce a number of potent angiogenic and lymphangiogenic growth factors, cytokines, and proteases, all mediators that potentiate neoplastic progression (50). Moreover, chemokines are potent activators of proteases that facilitate macrophage-mediated digestion of extracellular matrix (51, 52). In particular, CCL2 potently activates gelatinase, uPA, and its receptor (51, 53). The tissue-type plasminogen activator, uPAs, and their respective receptors, annexin II and u-PAR, were demonstrated to contribute to the invasive behavior of pancreatic cancer (54, 55). MMP-2 expression appeared to be an important determinant of pancreatic cancer dissemination as levels of active MMP-2 strongly correlates with nodal status and tumor stage (56). Thus, CCL2 may indirectly contribute to pancreatic cancer dissemination by favoring the leukocyte-mediated digestion of extracellular matrix. The evidence that CCL2 was not associated with metastasis, lymph node involvement, vessel invasion, and histological differentiation of the tumor suggested that this chemokine is not relevant for the dissemination of pancreatic tumor. Others have proposed a dual role of CCL2 on tumor growth. CCL2 can be protective in some tumor models but destructive in others: murine colon carcinoma cells expressing CCL2 fail to metastasize when injected into mice (57), whereas other carcinoma cells show enhanced metastasis (58). Overexpression of CCL2 by tumor cells can lead to their destruction by an infiltrate of activated mononuclear cells (59–62). Moreover, even if many clinical studies suggest that TAM are a part of inflammatory circuits that promote tumor progression (31, 34), other clinical evidences indicated that inflammation and chemokines may also impair tumor growth and metastasis (63). A recent careful analysis of the impact of CCL2 on tumor growth in a nontumorigenic melanoma system revealed a biphasic effect (64). Low-level CCL2 secretion, with physiological accumulation of TAM, promoted tumor formation, whereas high CCL2 secretion resulted in massive macrophage infiltration into the tumor mass and its destruction.

**A**

**B**

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cytokines induce pancreatic cancer apoptosis and inhibit proliferation remains to be clarified. The direct antitumor properties of proinflammatory cytokines are generally considered to reside in their ability to inhibit tumor growth (66) or cause cell death (67). Recently, it was suggested that TNF-α could inhibit proliferation of pancreatic cancer cells by increasing the production of superoxide anion (68). Moreover, in response to cytokine stimulation (TNF-α, IFN-γ, and IL-1β), human pancreatic carcinoma cell lines expressed the inducible NO synthase that synthesizes NO (40). NO is able to induce G1 arrest followed by apoptosis in some pancreatic carcinoma cell lines (40). A new model by which TNF-α and IL-1β may impair cell cycle progression was also recently suggested for breast cancer cells (69). In this scenario, proinflammatory cytokines impair exit from G0-G1 and reduce survival primarily by inhibiting autocrine or paracrine growth factor signals (i.e., insulin-like growth factor I) rather than directly suppressing cellular growth.

On the basis of the results of our study, tumor derived CCL2 has two major functions: (a) the chemotactic activity of CCL2 could be sufficient to start the recruitment of monocytes/macrophages to the tumor site where they are activated by other factors to secrete proinflammatory cytokines that lead to apoptosis of tumor cells and stimulate additional CCL2 secretion. Thus, high levels of CCL2 may reflect the presence of inflammatory cytokines at the tumor site. (b) CCL2 increases inducible NO synthase in monocytes and NO, as previous published, and is able to induce G1-arrest followed by apoptosis in pancreatic carcinoma cell lines (40, 70). This suggests that the role of CCL2 is not limited to macrophage recruitment but extends to a direct activation of monocyte-driven apoptosis of pancreatic tumor cells.

A hypothetical model may be suggested, illustrating the complex interaction between CCL2 expression by pancreatic cancer and the tumor microenvironment. According to this model, a prime event taking place in the early stages of pancreatic cancer development may be the ability to express CCL2, which will recruit circulating monocytes at the tumor site. Thereafter, as suggested by the analysis on regulation of CCL2 expression, proinflammatory cytokines secreted by infiltrating leukocytes or by other stromal cells may up-regulate CCL2 expression by tumor cells. Increased CCL2 expression amplies monocyte migration to the tumor and NO production. Overall, our results suggest that CCL2 and the cellular products that may be induced by its indirect effects might have a significant role in pancreatic cancer progression. The ability of CCL2 to induce processes that oppose proliferation and increase pancreatic cancer apoptosis is a novel, partially unexpected result and is not in full agreement with the CCL2 role observed in other cancer (e.g., breast and ovary cancer). The specific role and contribution to disease progression by tumor-infiltrating leukocytes and chemokines may be different depending on the tumor type. This underlies that the biological significance of the presence of chemokines in the tumor microenvironment is particularly complex and their ability to regulate cancer development has not been completely clarified.

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The CC Chemokine MCP-1/CCL2 in Pancreatic Cancer Progression: Regulation of Expression and Potential Mechanisms of Antimalignant Activity

Paolo Monti, Biagio Eugenio Leone, Federica Marchesi, et al.


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