Endoneurial Macrophages Induce Perineural Invasion of Pancreatic Cancer Cells by Secretion of GDNF and Activation of RET Tyrosine Kinase Receptor

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Conflict of interest
The authors declare no conflict of interest.
ABSTRACT

Perineural invasion of cancer cells (CPNI) is found in most patients with pancreatic adenocarcinomas (PDAs), prostate or head and neck cancers. These patients undergo palliative rather than curative treatment due to dissemination of cancer along nerves, well beyond the extent of any local invasion. Although CPNI is a common source of distant tumor spread and a cause of significant morbidity, its exact mechanism is undefined. Immunohistochemical analysis of specimens excised from patients with PDAs showed a significant increase in the number of endoneurial macrophages (EMΦs) which lie around nerves invaded by cancer compared to normal nerves. Video microscopy and time-lapse analysis revealed that EMΦs are recruited by the tumor cells in response to colony stimulated factor-1 secreted by invading cancer cells. Conditioned medium (CM) of tumor-activated EMΦs (tEMΦs) induced a 5-fold increase in migration of PDA cells compared to controls.

Compared to resting EMΦs, tEMΦs secreted higher levels of glial-derived neurotrophic factor (GDNF), inducing phosphorylation of RET and downstream activation of extracellular signal-regulated kinases (ERK) in PDA cells. Genetic and pharmacologic inhibition of the GDNF receptors GFRA1 and RET abrogated the migratory effect of EMΦ-CM and reduced ERK phosphorylation. In an in-vivo CPNI model, CCR2-deficient mice which have reduced macrophages recruitment and activation showed minimal nerve invasion, while wild-type mice developed complete sciatic nerve paralysis due to massive CPNI. Taken together, our results identify a paracrine response between EMΦs and PDA cells which orchestrates the formation of cancer nerve invasion.
EDITED PRÉCIS: A paracrine response between pancreatic adenocarcinoma cells and macrophages that rove nerve tracks appears to orchestrate nerve invasion by localized tumors, a type of invasion that occurs in various types of encapsulated glandular tumors.
INTRODUCTION

Solid tumors disseminate in four main ways: direct invasion, lymphatic spread, hematogenic spread, and through nerves. The spread of cancer cells along nerves is a frequent pathologic finding and a significant cause of morbidity and mortality (1), conferring poor prognosis to patients with carcinomas of the gastrointestinal tracts, head and neck, pancreas, and prostate (2). In the case of pancreatic ductal adenocarcinoma (PDA), most patients undergo palliative treatment rather than curative surgery due to extra pancreatic spread resulting from cancer perineural invasion (CPNI). During the last year of life, these patients will also suffer from debilitating neuropathic pain due to perineural cancer spread (3).

Certain tumors present with profound neural invasion at an early disease stage, whereas other highly aggressive tumors do not infiltrate nerves even at an advanced stage (4). Therefore, it is plausible that the propensity of a tumor to invade nerves is dependent on the specific properties of the cancer cell and on its interaction with the neural stroma. The perineural microenvironment includes neurons, Schwann cells, and microglia/macrophages which secrete factors that participate in nerve homeostasis, dendritic growth and axonal sprouting. The same host cells can also express soluble proteins that can potentially initiate and sustain cancer invasion (5). Previous observations led to the hypothesis that the perineural stroma can support tumor cell proliferation and dissemination (6). We and others have shown that glial-derived neurotrophic factor (GDNF), which normally promotes survival and differentiation of axons and nerves, can also promote CPNI (7-9). The ligand-binding component of GDNF is a glycosyl-phosphatidylinositol–anchored GDNF family receptor α-1 (GFRα1), which associates with its transmembrane co-receptor RET after ligand binding (10).
RET is a tyrosine kinase receptor and its phosphorylation promotes reorganization of actin dynamics and modulation of filopodia and lamellipodia formation (11), inducing migration and proliferation of epithelial cells (12), neurons (13) and cancer cells (14). Several members of the GDNF family of ligands and their receptors are up-regulated in many cancer cells, and their increased expression is associated with malignant progression and a poor prognosis (15-17). The exact mechanism by which the perineural environment facilitates dissemination through nerves is, however, still largely unknown.

Endoneurial macrophages (EMΦs) form a subpopulation of microglia/macrophages that participates in cellular defense and regeneration of peripheral nerves (18). At the tumor microenvironment, these immune cells can produce growth factors that promote cancer proliferation and invasion (19).

Here we report the first demonstration of macrophages infiltrating the perineural space in specimens derived from patients with PDA and in animal models. After their activation by cancer cells, EMΦs secreted high levels of GDNF which phosphorylate RET receptors on PDA cells promoting CPNI.

MATERIALS AND METHODS

Cell lines and reagents

Carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). The microglia cell line BV2 was acquired from the Weizmann Institute (Rehovot, Israel) and characterized in our laboratory as F4/80+ CD11b+ cells. Freshly dissociated EMΦs/microglia were prepared as described previously using Tg(CAG-EGFP)B5Ngy/J mice (7, 20). Over 90% of the population of EMΦs was confirmed by immunofluorescence and flow cytometry analysis using anti-CD11b and F4/80 antibodies (BD Biosciences, Bedford, MA).
For the generation of human macrophages, mononuclear cells from the blood of healthy donors were isolated by Ficoll centrifugation and incubated in 12-well plates for 2 h as described previously (21). The adherent monocytes were then incubated for 7 days in medium with macrophages colony stimulated factor in order to become resting macrophages.

Small molecule inhibitors, including U0126, PD98059 and pyrazolo-pyrimidine-1, were purchased from Biomol (Plymouth Meeting, PA). The following antibodies were used: P-ERK1/2, ERK1/2, F4/80, CD11b (Cell Signaling, Danvers, MA), β-actin, RET (C-19, sc-167), GFRα1 (Santa Cruz Biotech, Santa Cruz, CA), CD68, S100, nonimmune rabbit immunoglobulin (Calbiochem, La Jolla, CA), CD163, CD86 and P-RET monoclonal (R&D Systems, Minneapolis, MN).

For preparation of conditioned media (CM), cells were incubated for 48 h in serum free culture media before their medium was collected. In some experiments, resting macrophages/EMΦs were cultured for an additional 48 h alone or with CM from MiaPaCa2 or Panc2 cancer cells (of human or mouse origin, respectively) to generate tumor-activated macrophages/EMΦs.

Lipopolysaccharide (LPS)-enriched CM was prepared by incubation of EMΦs for 48 h in the presence of 1μg/ml LPS. Normal media containing 1mcg/ml LPS was used as control in these experiments. Migration assays and immunoblotting was performed as described (22). Small interfering RNA (siRNA) oligonucleotides directed against human GFRα1 (Stealth Select RNAi™) were custom-designed and validated by Invitrogen (Carlsbad, CA). Cells were transfected with three different transcripts of GFRα1 siRNA or with a non-targeting siRNA.

The dorsal root ganglion (DRG) model is based on a technique originally described for prostate cancer cells (23) and modified by our group (7). DRG-
derived EMΦs were identified according to their typical morphology, amoeboid motility and expression of the microglia/macrophages marker F4/80 (24). The concentration of GDNF was determined by a double-ligand ELISA according to the manufacturer’s protocol (Quantikine; R & D Systems) with test and control samples. cDNA PCR amplification was carried out on a Step One Plus Real Time PCR system (Applied Biosystems) with gene-specific oligonucleotide pairs (Sigma Aldrich, Israel). Each sample was analyzed in triplicate. Results were normalized to ACTB and 18S mRNA levels.

**Mice**

Wild-type C57BL/6 female breeders, CCR2<sup>−/−</sup> mice (strain B6.129S4-Ccr2<sup>2m1Ifc</sup>/J), and transgenic GFP-CETN2 mice that express an enhanced green fluorescent protein-labeled human Centrin-2 (Tg(CAG-EGFP)B5Ngy/J; 2–4 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Maine).

**Statistics**

The patient population used for immunostaining analysis included randomly selected cases of PDA identified through a search of the Tel Aviv Sourasky Medical Center. The non-parametric Mann–Whitney U test was used for analysis of variance. Fisher's exact test was used in the analysis of qualitative data. All P values were calculated using two-sided tests using the Origin statistical package (OriginLab Corporation, Northampton, MA). Differences were considered significant if P was less than 0.05. Error bars in the graphs represent standard deviation. All experiments were repeated at least three times.
RESULTS

Macrophages are a prominent stromal component of the perineural cancerous environment

A host stromal response to an invasive PDA involves the infiltration of a variety of inflammatory cell types, including macrophages (25). To explore the stromal response to CPNI, we compared the patterns of macrophage infiltration around nerves in pathological specimens excised from ten patients with PDA. The degree of macrophages infiltration around intra- and extra pancreatic nerves was studied using immunolabeling with the macrophages marker CD68 (Fig. 1). Infiltrating CD68-positive cells were noted around nerves invaded by cancer (mean=27±3 macrophages/nerve, Fig. 1a). In contrast, most nerves that were not invaded by cancer showed relatively low numbers of CD68-positive cells infiltration (mean=4±2; Fig. 1b, P<.001). Figure 1e-f shows high magnification pictures of CD68-positive cells around a nerve invaded by cancer. This finding, along with other studies that showed that macrophages are involved in cancer cell invasion (26) led to the hypothesis that EMΦ may play a role in CPNI.

Recruitment of endoneurial macrophages to the tumor invasion front

To further study the interactions between endoneurial macrophages we used an in-vitro model of CPNI. Mouse (Panc2) or human (MiaPaCa2) PDA cell lines were grown in matrigel adjacent to preparations of freshly dissociated cultured DRG. This model is frequently used for studying the paracrine interaction between neural stroma and cancer cells (2, 6, 7, 27, 28). Using video microscopy imaging, we noticed that cancer cells dissociated from their colony and formed clusters that migrated in a unidirectional fashion along the nerve
toward the ganglion (Fig. 2a). Over time, these cells formed bridgeheads to facilitate more extensive polarized, neurotrophic migration of cancer cells (Fig. 2b). Cancer cells did not disperse from their colony in areas without nerve contact and therefore invasion occurred only in regions adjacent to nerves. We next focused our attention on the interactions between PDA and DRGs before the initiation of CPNI. Live fluorescent microscopy imaging of EMΦs expressing green fluorescent protein (GFP) and of PDA cells expressing red fluorescent protein (RFP) revealed the close interactions between the two populations of cells (Fig. 2c). Further time-lapse analysis revealed that rapid recruitment of resident GFP+ cells occurred hours before the onset of neural invasion. These EMΦs were recruited to the tumor invasion front within several hours, much before direct axon-tumor contacts were formed (Fig. 3a). Direct cell–cell contacts were frequently observed between EMΦs and PDA cells (Fig. 3b). These contacts had an extended duration and led to a dramatic increase in the dwelling time and number of EMΦs at the tumor invasion front (Fig. 3c, n=12). Most importantly, detailed examination of the time-lapse data revealed that after their recruitment to the tumor invasion front, EMΦs rarely migrated away from the tumor, while EMΦs located at a distance from the tumor showed no consistent directional path. Despite their close interaction with the tumor cells, EMΦs did not prevent the migration of PDA cells towards the DRG. In order to further investigate whether EMΦ recruitment is induced by soluble proteins secreted by the cancer cells, we used a transwell migration assay in which conditioned media (CM) from PDA cells (MiaPaCa2 or Panc2) was added to the lower chamber and the EMΦs were plated on the insert. PDA-CM induced a significant increase in migration of human macrophages and murine EMΦs, respectively, compared to control medium (Fig. 3d). We then evaluated
the ability of EMΦs to migrate in a matrigel matrix towards a colony of PDA cells. Similar to the two previous assays, migration of EMΦs was more prominent towards the cancer colony than to the opposite direction (supplementary Fig. 1a-c). Taking together, these findings indicate that EMΦs are recruited to the tumor front by a soluble protein(s) secreted form cancer cells.

**Migration of EMΦs towards PDA is induced by CSF-1**

In an initial effort to characterize the proteins secreted by PDA cells that may be involved in EMΦ recruitment, we determined the relative expression level of 32 cytokines, chemokines and growth factors expressed by MiaPaCa2 cells. Analysis revealed >3-fold increase in expression of colony-stimulating factor-1 (CSF-1) relative to baseline. Expression of CSF-1 by PDA cells was confirmed by RT-PCR ([Fig. 3e](#)), inset. CSF-1 controls the survival, proliferation, migration and differentiation of mononuclear phagocytes and appears to play a role in cancer progression (29). Previous reports have also indicated that PDA secretion of CSF-1 participates in the recruitment of tumor associated macrophages (30, 31). In light of these findings, we investigated whether CSF-1 and its receptor CSF-1R, are involved in EMΦ migration towards the PDA-CM. GW2580 is a selective small molecule kinase inhibitor of CSF-1R (32). Using transwell migration assays we found that this competitive inhibitor of adenosine triphosphate binding prevented PDA-dependent migration of macrophages in a dose dependent manner ([Fig. 3e–blue bars](#)), but had a negligible effect on their migration in the absence of PDA-CM ([Fig. 3e- red bars](#)). It was shown that macrophages activation by CSF1 is dependent on ERK phosphorylation (33). To identify the mechanism by which CSF1 secreted by PDA cells activates EMΦ, we tested whether blocking CSF-1R by GW2580, reduces ERK activation. Figure 3f
shows western blotting analysis of EMΦ in different conditions. This analysis revealed that stimulation of EMΦ by PDA-CM induced phosphorylation of ERK, and that inhibition of CSF-1R or MEK-1 by GW2580 or PD98059 respectively, decreased downstream ERK activation.

We further characterize those macrophages according to the M1 (classically activated), M2 (alternatively activated) terminology, by testing the expression of CD86 and CD163, typical M1 and M2 markers, respectively.(34) Monocyte-derived human macrophages were incubated with M-CSF, LPS, or condition media of MiaPaCa2. **Supplemental Figure 2** shows that incubation with LPS resulted in pure M1 population (**Supplemental Fig. 2a**) as oppose to M-CSF, which produced M2 population (**Supplemental Fig. 2b**). Interestingly incubation with MiaPaCa2-CM resulted in mixed M1/M2 population (**Supplemental Fig. 2c**), suggesting that these activated macrophages form a continuum of phenotypes between purely M1-classified and M2-classified populations (35).

**EMΦs induce paracrine signals that initiate PDA cell migration**

Having observed that PDA cells become spindle-like and polarized towards the DRG even without direct contact, we reasoned that it is possible that EMΦs that reside around nerves invaded by cancer are secreting a factor(s) that induces CPNI. To study how EMΦs alter the migratory behavior of PDA cells we used migration assays where EMΦ-CM was added to the lower chamber as the chemoattractant, and MiaPaCa2 or Panc2 cells were added to the insert. PDA cells demonstrated 2-fold increased migration towards resting EMΦ (rEMΦ) compared to control media (**Fig. 4a**, black bars, P<.001, n=50). Interestingly, one log dilution of the CM still resulted in significant migration compared to
control (n=10; P<.001). While EMΦs induced a significant increase in migration of PDA cells, they had no effect on non-neurotrophic cells, including squamous cell carcinoma (QLL2) or melanoma (B16F10) cell lines (data not shown). Similarly, CM of PDA cells had a minimal effect on migration of pancreas cell lines (Fig. 4a).

Wound-healing assay was used to further evaluate the effect of EMΦ-CM on PDA cell motility. MiaPaCa2 cells were plated in normal medium for 3 hours and then incubated overnight in serum free media. After the scratch was performed, the cells were exposed to EMΦ-CM or to normal media for another 24 hours. Cancer cells exposed to EMΦ-CM migrated more rapidly to close the scratched wound compared with those exposed to culture media alone (supplementary Fig. 3a and b). These findings indicate that EMΦs release soluble factors that promote cellular motility of PDA cells.

Activated EMΦs augment cancer cell migration

Having found that resting rEMΦ CM induces cancer cell migration, it was important to investigate whether activated EMΦs can enhance the migratory effect on PDA cells. Towards this end, we stimulated the primary murine EMΦs with lipopolysaccharide (LPS), or with Panc2-CM (tEMΦ). CM from LPS or tEMΦ was added to lower chambers of transwell plates as the chemoattractants. Figure 4a shows that tEMΦ-CM significantly augmented the migration of PDA cells compared to Panc2-CM or rEMΦ-CM alone (P<.001, n=10). The effect of tEMΦ-CM on migration was larger than the combined effect of Panc2-CM and rEMΦ-CM (for the additive effect, see arrow in Fig. 4a). Similar but slightly lower effect was found when the EMΦs were stimulated by LPS.
To confirm our observations, we repeated these experiments using blood-borne human macrophages (hMΦ) and the human PDA cell line MiaPaCa2. **Figure 4b** shows that CM from hMΦ significantly increased the migration of MiaPaCa2 cells relative to normal medium or MiaPaCa2-CM. Again, the effect of tumor-activated macrophages was significantly larger than that of resting macrophages or of the effect of MiaPaCa2-CM and resting macrophages combined (**Fig. 4b** arrow, n=10).

In another set of experiments we studied the effect of CM from a macrophages/microglia cell line (BV2) which resembles EMΦ (36). In agreement with our previous results, BV2-CM induced a significant increase in migration of PDA cells compared to controls ($P<0.001$, $n=24$, data not shown).

**EMΦs induce PDA migration by secretion of GDNF and activation or RET**

Immunofluorescent imaging of PDA specimens revealed expression of the GDNF receptor GFRα1 and its co-receptor RET by cancer cells in all tested specimens (**supplementary Fig. 4a-h**, n=15). Since PDA cells express GDNF receptors, and given that previous data suggested that GDNF is involved in CPNI, we investigated the secretion of GDNF by resting EMΦs, tumor-activated EMΦs and LPS-activated EMΦs. Activation of EMΦs by LPS or PDA-CM triggered a >50% increase in GDNF secretion relative to resting EMΦs (**Fig. 5a**). This finding is in agreement with previous data showing an increase in GDNF secretion by activated EMΦ (37-40).

Since recombinant human GDNF induced migration of MiaPaCa2 cells at similar doses (**fig. 5b**), we further investigated the involvement of the GDNF receptor GFRα1 in PDA cells migration by using small interfering RNA (siRNA) oligonucleotides directed against expression of GFRα1 (**Fig. 5c**) MiaPaCa2 cells.
transfected with siGFRα1 exhibited a significant decrease in cancer cell migration compared to non-coding siRNA (Fig. 5d). Consistent with these findings, treatment of PDA cells with pyrazolopyrimidine-1 (PYP1), a potent RET inhibitor (41), also effectively inhibited cancer cell migration toward EMΦ-CM (Fig. 5e). Since PYP1 had no effect on cancer cell proliferation (Supplemental Fig. 5), it is possible that the effect of PYP1 was much stronger than the effect of siGFRα1 due to incomplete suppression of siRNA, leading to residual signaling.

To further investigate the ability of EMΦs to activate RET receptors on PDA cells, we studied the ability of CM from EMΦs to phosphorylate RET receptors by western blotting. As shown in figure 5f, EMΦ-CM induced phosphorylation of RET within several minutes, which decayed 30 minutes after the CM was added. Taken together these data indicate that GDNF secretion from EMΦs activates RET tyrosine kinase receptors on PDA cells and induces their migration.

**EMΦ-secreted GDNF induces activation of RET signaling pathways**

To identify the downstream signals that mediate GDNF-induced PDA migration, MiaPaCa2 cells were incubated for 20 min with EMΦ-CM and the phosphorylation level of ERK was determined by immunoblotting. Figure 6a shows that MiaPaCa2 cells induced by EMΦ-CM had significantly higher levels of phospho-ERK compared with controls. Furthermore, inhibition of RET or MEK-1 effectively blocked ERK phosphorylation (Fig. 6a). Treatment of MiaPaCa2 cells with potent inhibitor of MEK-1 also partially suppressed EMΦ-induced migration (Fig 6b). This inhibitory effect was not due to decrease in the number of cells as examined by XTT assay (data not shown). The partial suppression of EMΦs-induced migration by MEK-1 inhibitors suggested to us that other signaling
pathways may be involved in this effect. Therefore we studied the involvement of the PI3K pathway in EMΦ-induced migration. Figure 6c shows that inhibition of AKT by LY294002 also partially reduced cancer cell migration towards the EMΦ-CM. This result suggests that the PI3K pathway is also involved in this process.

**Reduced CPNI in CCR2-deficient mice**

Finally we sought to investigate the impact of macrophages on CPNI by using a nerve invasion model in-vivo. We used the sciatic nerve invasion model, which was shown to reliably represent nerve invasion in mice (42). The endoneurial macrophages (producing GDNF) of the sciatic nerve are located in the dorsal root ganglia, so our previous experiments with EMΦs are representative of the in-vivo murine sciatic nerve model. Here, nerve invasion was investigated in CCR2-/- mice which have reduced recruitment and activation of tumor-associated macrophages (43) and in wild type (WT) controls. Murine Panc2 PDA cells were implanted in a distal part of the sciatic nerve and their ability to invade along the nerve toward the dorsal root ganglia was investigated histologically and physiologically. Figure 7 shows the difference in kinetics of hind limb dysfunction as a result of sciatic nerve invasion in WT and CCR2-deficient mice. Of the 14 nerves studied in the WT group, 13 were fully paralyzed by day 7 (Fig. 7a-c). In contrast, only 4 of the 12 nerves in the CCR2-/- group showed mild degree of nerve paresis (P=.003). The rest 8 mice in the CCR2-/- group had normal nerve function (Fig. 7d).

All mice were euthanized at day 8 and their sciatic nerves were excised for histopathologic analysis. Immunofluorescence staining with anti F4/80 Ab revealed high numbers of EMΦs in tumors of WT mice but not in CCR2-/- mice.
(Fig. 7e). The degree of nerve invasion was determined by measuring the nerve diameter ~3 mm proximal to the implantation site, along the sciatic nerve. The mean diameter of control nerves injected with saline was 0.3±0.01 mm, and the mean diameter of nerves from WT mice was 0.72±0.1 mm, significantly larger than that of CCR2-/- mice (0.35±0.03 mm; n=7 in each group, *P*<.001), due to massive cancer invasion (Fig. 7f and g). CPNI was verified with immunohistochemical staining and with hematoxylin and eosin staining.

These data suggest a paracrine loop between EMΦs and PDA cells as summarized in Figure 7h.

**DISCUSSION**

Nerve invasion by cancer is emerging as an important clinical problem in many malignancies, including those of the pancreas. However, despite increasing recognition of this common metastatic process it has received relatively little research attention. Ketterer et al., have demonstrated using laser capture analysis that neurotrophic growth factors and their receptors are abundant in nerves with perineural invasion (6). The presence of neurotrophins receptors in the cancer cells, in conjunction with the ability of their ligands to modulate PDA cell growth as revealed by nerve-PDA co-cultures, suggested that a paracrine loop may exist between nerves and PDA cells (6).

In the current study, we expand on these findings in an effort to better delineate the involvement of stromal cells in CPNI. We focused on a subpopulation of macrophages/microglia located in the perineural space, which participates in cellular defense and nerve homeostasis. The evidence found in this study suggesting that macrophages contribute to perineural invasion is based on the following findings: 1) endoneurial macrophages are abundant in
nerves invaded by cancer, 2) endoneurial macrophages are recruited by cancer cells to the tumor front, 3) conditioned media from endoneurial macrophages induces cancer cell invasion, 4) activation of endoneurial macrophages by cancer cells triggers an increase in GDNF secretion, which then acts as a chemoattractant on cancer cells, 5) endoneurial macrophages induce activation of RET receptors on cancer cells and downstream activation of ERK, 6) knockout of GFRα1 by siRNA inhibits the effect of endoneurial macrophages on cancer cell migration, 7) pharmacologic inhibition of RET blocks the effect of endoneurial macrophages on cancer cell migration, 8) pharmacologic inhibition of MEK-1 and AKT blocks the effect of endoneurial macrophages on cancer cell migration, and 9) perineural invasion is decreased in CCR2-knockout mice which lack macrophages trafficking and activation. Similar to our findings, activation of ERK by GDNF-GFRα1-RET signaling was shown to induce cell migration in other systems, including corneal (44), epithelial (12), and nerve cells (45). The effect of GDNF can be augmented by RET activity (46), G691S RET polymorphism (9) and by an inflammatory response (15).

In our current study, we used the sciatic nerve invasion model and CCR2-deficient mice to expand on the mechanism of CPNI. The lack of CCR2 expression resulted in fewer EMΦs at the perineural area and diminished CPNI. There are additional indications for the pro-tumoral properties of CCR2 in other cancers, which have been attributed to recruitment of TAMs (47, 48). Whereas these cells are recruited to sites of inflammation through CCR2 signaling, it seems that local factors, such as CSF-1, recruit macrophages to the tumor periphery where they secrete motility factors that facilitate tumor cell invasion (31). These findings along with studies performed in conventional Boyden chamber assays suggest the existence of paracrine interactions between PDA
cells and endoneurial macrophages. We do not claim that these models summarize the whole spectrum of the human disease, but we believe that they can represent the potential of mutual interaction between neural stroma and cancer cells. Further studies are indeed required to examine the clinical relevance of this finding, preferably by using genetically engineered cancer-prone mice (49). In addition, other proteins including adhesion molecules, cytokines, metalloproteinases, cathepsins and/or additional growth factors are likely to be involved in this process (50).

In conclusion, this study suggests the existence of a paracrine loop between tumor-infiltrating EMΦs and pancreatic cancer cells that contributes to CPNI. Tumor-associated EMΦs are activated by soluble factors secreted by tumor cells which then trigger secretion of GDNF. The proinvasive/promotile capabilities of EMΦ-secreted GDNF are achieved by RET signaling induced activation of MAPK and PI3K pathways in cancer cells. Characterization of this paracrine loop has important clinical implications, since it provides pharmacological targets which may be intersected to reduce neuroinvasion of PDA. Treatment directed against CPNI could prevent cancer spread, prolong survival and reduce morbidity. It can be offered as an adjuvant therapy to enhance conventional cytotoxic strategies, or used to prevent CPNI independently of its ability to reduce the size of the primary tumor.
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REFERENCES


FIGURE LEGENDS

Figure 1. Macrophage infiltration around intrapancreatic nerves in pathological specimens excised from patients with pancreas cancers.

(a) Low magnification (X20) immunohistochemical staining with anti-CD68 antibody (macrophages/microglia marker) showing a nerve invaded by cancer (see high magnification pictures in e' and f') and (b) a normal nerve. (c) Immunohistochemical staining with anti-S100 antibody (nerve marker) of the specimen shown in a'. (d) Staining with anti-S100 antibody of the specimen shown in b'. (e) and (f) high magnification (X40) immunohistochemical staining with anti-CD68 antibody showing macrophages infiltration around nerve invaded by cancer. N - indicates nerve's bundle, arrows - islands of invading tumor cells; positive staining is shown in brown.

Figure 2. Nerve invasion of pancreas carcinoma cells in a dorsal root ganglion model. (a) Invasion of the ganglionic nerve 7 days after implantation (magnification X10). (b) A picture of the same area taken 3 days later showing extensive nerve invasion by cancer cells. The yellow arrows indicate the area of the axons invaded by cancer (magnification X10). The illustration on the right depicts the position of tumor colony and ganglionic nerves. White asterisk-indicates the tumor colony and green asterisk the ganglion. (c) Live fluorescent microscope image (magnification X20) taken several hours before nerve invasion was initiated shows close interaction between macrophages (GFP-expressing) and cancer cells (RFP-expressing).
Figure 3. Recruitment of endoneurial macrophages to the tumor front.  
(a) Phase contrast (left) and fluorescent microscope (right) pictures showing presence of GFP-positive EMΦs at the tumor front (magnification X20). The dotted black box indicates the area of the picture on the right. (b) Video microscopy pictures showing fast recruitment of GFP-positive EMΦs before neural invasion is initiated (magnification X40). The dotted black box indicates the area of the picture on the right. (c) Time-lapse analysis revealing a increase in the number of EMΦs at the tumor invasion front (blue bar) compared to the area without tumor cells (red bar). (d) Transwell migration assay in which conditioned media (CM) from Panc2 cancer cells (Panc2-CM) was added to the lower chamber and the EMΦs were plated on the insert. Panc2-CM (blue bar) induced a significant increase in migration of EMΦs compared to control (DMEM) medium (red bar). (e) Migration of human macrophages towards MiaPaCa2-CM with or without CSF-1 receptor blocker GW2580. Red bars indicate media without MiaPaCa2-CM and blue bars indicate the addition of MiaPaCa2-CM (*P=0.01, **P<0.001, n=12). Inset- expression of CSF-1 ligand by PDA cancer. (f) Western blotting analysis of EMΦ in different conditions. Activation of EMΦs by PDA-CM resulted in phosphorylation of ERK, while inhibition of CSF-1R (GW2580) or MEK-1 (PD98059) blocked PDA-induced ERK phosphorylation.

Figure 4. Migration of pancreatic carcinoma cells induced by macrophages-CM. (a) Resting EMΦ-CM (rEMΦ-CM) increased the migration of Panc2 cells relative to normal media. Activation of rEMΦ by LPS (lEMΦ) and tumor activated EMΦ (tEMΦ) further increased pancreatic cancer cells migration. The arrow indicates the sum effect of PDA-CM and rEMΦ-CM (n=10-40). (b) Migration of cancer cells towards CM of human macrophages (hMΦ). Resting
hMΦ-CM (rhMΦ-CM) increased MiaPaCa2 cells migration relative to normal (control) medium (n=10) and tumor activated hMΦ (thMΦ) CM synergistically augmented the migratory effect on cancer cells relative to rhMΦ-CM and MiaPaCa2-CM combined (indicated by the arrow).

Figure 5. EMΦ-induced pancreatic cancer cell migration is mediated by GDNF. (a) GDNF levels secreted by resting EMΦ (rEMΦ), LPS-activated EMΦ (lEMΦ) and tumor activated EMΦ (tEMΦ) revealed by ELISA (n=5 experiments per condition). (b) Recombinant human GDNF (rGDNF) induces migration of MiaPaCa2 cells in Boyden chamber assays (n=5 in each dose). (c) Immuncytochemistry with anti-GFRα1 antibodies, of MiaPaCa2 cells transfected with siRNA directed against GFRα1 (magnification X20). Left panel - siControl, right panel - siGFRα1. (d) MiaPaCa2 cells transfected with siGFRα1 exhibited a significant decrease in cancer cell migration towards the EMΦ-CM compared to siControl. There was no effect of siGFRα1 on cancer cell migration when normal medium was used instead of EMΦ-CM (n=6 in each condition). (e) Treatment of pancreatic adenocarcinoma cells with pyrazolopyrimidine-1 (PYP1 2μM), a potent RET small molecule inhibitor, effectively inhibited cancer cell migration toward EMΦ-CM (gray bars) but had no significant effect when normal medium (black bars) was used instead (n=8 in each experiment). (f) Western blot analysis showing an increase in phospho-RET minutes after adding of EMΦ-CM. Shown below it is a densitometry analysis from 3 experiments (*- P = 0.005).

Figure 6. Downstream signaling events in PDA cells following stimulation with EMΦ. (a) MiaPaCa2 cells were incubated for 20 min with EMΦ-CM and the phosphorylation level of ERK was determined by western
blotting. EMΦ-CM induced phosphorylation of ERK, while treatment with the RET inhibitor (PYP1) or the MEK-1 inhibitor (PD98059) effectively blocked ERK phosphorylation. (b) Inhibition of MEK-1 (U0126 10 µM) partially suppressed EMΦ-induced migration (n=6, \( P < .001 \)). (c) Inhibition of AKT (LY294002) suppresses EMΦ-induced migration (n=6, \( P < .01 \)).

**Figure 7. Reduced sciatic nerve invasion in CCR2-deficient mice in-vivo.**

(a) Representative image of the hind-paw function of a WT mouse 7 days after tumor implantation, showing right limb paralysis. (b) Hind-limb function of a CCR2-/- mouse showing normal function. (c) Mean sciatic nerve function score of WT and of CCR2-/- mice. A score of 5 indicates normal function, while a score of 1 indicates total hind limb paralysis (\( P = .003 \)). (d) Box plot showing the mean sciatic nerve function score in WT (red) and CCR2-/- (green) mice at day 7. The box is determined by the 25th and 75th percentiles. The whiskers are determined by the 5th and 95th percentiles (\( P = .003, n=8 \) per group). (e) Representative immunohistochemical staining pictures of the intraneural tumors with anti-F4/80 antibody (red- a macrophage marker), and anti-CD3 antibody (green- a T-cells marker CD3) at day 7. Left- WT mouse and right- CCR2-/- mouse (magnification X40). (f) Nerve invasion by cancer in a WT mouse (left) and in a CCR2-/- mouse (right, magnification X4). showing massive invasion of PDA cells in the WT mouse compared to CCR2-/- mouse. (g) The proximal diameter of the sciatic nerve was significantly smaller in the CCR2-/- group compared with the WT group (\( P < 0001 \)). Each dot represents one mouse, whereas the mean values are indicated by the open circles. (h) A canonical model of the paracrine loop between endoneurial macrophages and pancreatic ductal adenocarcinoma cells. CSF-1 secretion by cancer cells mediates
chemotaxis of endoneurial macrophages by activation of CSF-1 receptors (CSF-1R). EMΦs recruited to the tumor front are activated by the cancer cells and secrete GDNF which activates GFRα1/RET receptors on cancer cells inducing cancer nerve invasion.
Endoneurial Macrophages Induce Perineural Invasion of Pancreatic Cancer Cells by Secretion of GDNF and Activation of RET Tyrosine Kinase Receptor

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