Background: Aggressive breast tumor cells secrete platelet-derived growth factor C (PDGF-C).

Results: PDGF-C prevents staurosporine-induced macrophage apoptosis through PI3K/Akt activation and Bad phosphorylation, resulting in the inhibition of caspase activation and PARP cleavage.

Conclusion: PDGF-C has an anti-apoptotic effect on macrophages.

Significance: PDGF-C secreted from malignant tumor cells could affect the survival of tumor-associated macrophages.

PDGF-C, which is abundant in the malignant breast tumor microenvironment, plays an important role in cell growth and survival. Because tumor-associated macrophages (TAMs) contribute to cancer malignancy, macrophage survival mechanisms are an attractive area of research into controlling tumor progression. In this study, we investigated PDGF-C-mediated signaling pathways involved in anti-apoptotic effects in macrophages. We found that the human malignant breast cancer cell line MDA-MB-231 produced high quantities of PDGF-C, whereas benign MCF-7 cells did not. Recombinant PDGF-C induced PDGFR α chain phosphorylation, followed by Akt and Bad phosphorylation in THP-1-derived macrophages. MDA-MB-231 culture supernatants also activated macrophage PDGFR-α. PDGF-C prevented staurosporine-induced macrophage apoptosis by inhibiting the activation of caspase-3, -7, -8, and -9 and cleavage of poly(ADP-ribose) polymerase. Finally, TAMs isolated from the PDGF-C knockdown murine breast cancer cell line 4T1 and PDGF-C knockdown MDA-MB-231-derived tumor mass showed higher rates of apoptosis than the respective WT controls. Collectively, our results suggest that tumor cell-derived PDGF-C enhances TAM survival, promoting tumor malignancy.

The PDGF family comprises potent mitogens for cells of mesenchymal origin; these factors are also important regulators of cell migration, growth, survival, apoptosis, and transformation (1–3). Although PDGF is synthesized, stored, and released by platelets upon activation, it is also produced by a plethora of other cells, including smooth muscle cells, activated macrophages such as alveolar macrophages, and vascular endothelial cells (4–8). In mammals, four PDGF genes (A, B, C, and D) are currently known (9–12). Among them, PDGF-CC (PDGF-C homodimer) is the third member of the PDGF family (10, 13). PDGF-C is secreted as an inactive form, which requires proteolytic cleavage of the CUB (complement C1r/C1s, Uegf, Bmp1) domain for the growth factor domain (GFD)3 to bind and activate the PDGF receptor α (PDGFR-α) homodimer or PDGF receptor α/β (PDGFR-α/β) heterodimer (13). Recent findings indicate that PDGF-C is present in many breast cancer microenvironments at high levels and is related to prognosis (3). Tumor-derived PDGF ligands are thought to function in both an autocrine and a paracrine manner, activating receptors on tumors as well as surrounding stromal cells (3, 14, 15).

Solid tumors have been recognized as chronic inflammatory sites characterized by significant infiltration of leukocytes, various cytokines, and chemokines (16, 17). The tumor microenvironment contains not only resident cell types such as fibroblasts and pericytes but also migratory leukocytes such as neutrophils, mast cells, and, most notably, macrophages. The tumor microenvironment educates macrophages to promote tumor progression and metastasis (16, 18–22). Therefore, tumor-associated macrophages (TAMs) mostly exhibit the M2 phenotype and, thus, have been noted for their significant contribution to tumor progression and metastasis (23). Additionally, TAM-derived VEGF builds new blood and lymphatic vessels, allowing tumor cells to escape from the microenvironment (24, 25). Transforming growth factor β-1 secreted by TAM induces the transition of epithelial cells to mesenchymal cells in tumors, enhancing their invasiveness (26, 27). In addition, macrophage-derived matrix metalloproteases are involved in extracellular matrix degradation, enabling new pathways of cellular metastasis (28). An abundance of macrophages in the tumor mass is reported to be related to a poor prognosis (23); this might be a consequence of the interaction between macrophages and tumor cells. In other words, a
greater number of malignant cells can produce chemoattractants as well as survival signals for monocytes/macrophages; these leukocytes increase the levels of factors that enhance the aggressiveness of tumor cells.

To identify proteins that could affect TAMs, we reanalyzed previously published Gene Expression Omnibus profiles of the malignant human breast cancer cell line MDA-MB-231 and the benign breast cancer cell line MCF-7. PDGF-C synthesis was >40-fold higher in MDA-MB-231 than in MCF-7 cells. In preclinical models of human lung tumors, it was confirmed that PDGF-C recruits PDGFR-α-positive tumor fibroblasts, thereby promoting tumor growth (29). Similarly, PDGF-C autocrine signaling has also been observed in the initiation and progression of brain tumors such as glioblastoma and medulloblastoma (30, 31). Although PDGF-C production in invasive breast tumor patients is up-regulated (3), to date, no specific evidence regarding the role of PDGF-C on TAM is available.

In an effort to extend our understanding of the role of tumor-derived PDGF-C in the tumor microenvironment, and particularly its effects on macrophages, we assessed PDGF-C-mediated signaling pathways in THP-1 macrophages. Here, we report that PDGF-C mediates anti-apoptotic effects through Akt/Bad phosphorylation, underlining the importance of the survival mechanisms of macrophages resident in tumors.

**Experimental Procedures**

**THP-1 Cell Differentiation into Macrophages and Reagents**—A human leukemic cell line, THP-1, was obtained from ATCC (Middlesex, UK) and cultured in RPMI 1640 medium (HyClone, Logan, UT) supplemented with 10% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO2. THP-1 cells (1 × 10⁶/ml) were differentiated by 100 ng/ml PMA, Sigma-Aldrich) stimulation for 3 days and a further 5 days of resting after PMA removal (hereafter termed THP-1 macrophages). Recombinant human PDGF-C (R&D Systems, Minneapolis, MN) was dissolved in 4 mM HCl containing 0.1% BSA (BOVGEN, East Keilor, Australia) and treated at 100 ng/ml.

**Human Peripheral Blood Mononuclear Cell Isolation and Differentiation**—Human blood acquisitions were approved by the Institutional Review Board of Seoul National University, Korea (no. 1308-002-508). Differentiation day 5 and 7 human macrophages were fixed with 4% paraformaldehyde and permeabilized with BD Perm/Wash buffer (BD Biosciences) before antibody staining. Flow cytometry was used for flow cytometry. To detect macrophage marker expression, the fixed human macrophages were incubated with fluorescence-conjugated anti-human CD11b, CD14, CD68, and CD163 antibodies (eBioscience). All flow cytometry was performed using LSRII Green (BD Biosciences).

**Apoptosis Induction and Detection**—THP-1 macrophages were treated with 200 nM staurosporin (STS, Cayman Chemicals) and incubated for 3–8 h. Externalization of phosphatidylserine to the outer layer of the cell membrane was examined using the annexin V-FITC apoptosis detection kit I (BD Biosciences). Cells were washed, suspended in the Annexin V binding buffer, and stained with FITC-conjugated annexin V antibody and 7-aminoactinomycin D for 15 min at room temperature. Flow cytometry was performed using LSRII Green (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

**Concentration of Cancer Cell Culture Supernatants**—MDA-MB-231, MCF-7, HT29, A549, and MeWo cells were seeded in 100-π dishes at 1 × 10⁶ cells/ml. After 48 h, culture supernatants containing cancer cell-secreted proteins were collected, and 13–15 ml media were concentrated in a swinging bucket rotor at 3900 × g at 25 °C for 18–20 min using a 10-K Amicon
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Ultra-15 centrifugal filter (Millipore). Concentrated medium protein was assayed by Western blotting.

MTT Assay—Cell viability of THP-1 macrophages at resting day 7 was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. THP-1 cells (1 × 10⁶/ml) were differentiated in a 12-well plate with 100 ng of PMA stimulation for 3 days and a further 7 days of resting after PMA removal. 100 μl of MTT solution (5 mg/ml) in culture medium was added. Cells were then incubated further 4 h at 37 °C. Thereafter, medium was discarded, and cells were lysed in 1 ml of dimethyl sulfoxide. The absorbance of the resulting solutions was read at a wavelength of 560 nm in a VICTOR™ X3 microplate reader (PerkinElmer Life Science).

Western Blotting—Cells were lysed using radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 μM Na₂VO₄, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF) containing protease and phosphatase inhibitor mixture (GenDEPOT, Barker, TX). After brief sonication, samples were centrifuged at 13,000 rpm for 15 min and supernatants were collected. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Protein samples were resolved by denaturing 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then separated in 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Membranes were blocked in PBST and then incubated with antibodies (Cell Signaling) and horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich) for 2 h at room temperature. The membrane was washed three times and visualized using the Luminol reagent (ECL™ Prime).

Real-time PCR—For real-time RT-PCR, mRNA from MDA-MB-231, MCF-7, MeWo, A549, HT-29, and 4T1 cell lines was assayed in triplicate. cDNA was synthesized from 1 μg of total RNA using oligo(dT) primers and SuperScript reverse transcriptase (Invitrogen). The following primers were used for PCR analysis: human PDGF-C, 5′-GCCAGGTGTCTCTCGGT-TTA-3′ (forward) and 5′-CTTTGGGACACATTGACAT-3′ (reverse); human GAPDH, 5′-CGGAGTCACCGGATTGG-3′ (forward) and 5′-AGCCCTTCTCATGGTGTTG-3′ (reverse); murine PDGF-C, 5′-AGGTGTCTCTCGGTCAAGC-3′ (forward) and 5′-CCTGGGTTCACCTACAC-3′ (reverse); and murine GAPDH, 5′-TGGCTGAGGCAAGCTGAG-3′ (forward) and 5′-TGGCTGAGGCAAGCTGAG-3′ (reverse). The real-time PCR reagent was SYBR Green Master Mix (Applied Biosystems), and all PCR analyses were performed using an ABI Prism 7900HT (Applied Biosystems).

Animal Experiments—All animal procedures were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Seoul National University of Korea (approval no. SNU-130923-1). The 4T1 murine breast cancer cell line was obtained from ATCC and maintained in RPMI complete medium. The 4T1_PDGF-C knockdown cell line and MDAMB231_PDGF knockdown cell line were generated through shRNA lentiviral particle (Santa Cruz Biotechnology) infection followed by selection in 4 μg/ml puromycin for 2 weeks. Six-week-old female BALB/c mice were purchased from Orient Bio (Korea) and maintained in pathogen-free housing. NOD-SCID female mice were kindly provided by Dr. J. G. Park (Seoul National University, Seoul, Korea) at 6–7 weeks of age. For orthotopic implantation of tumor cells, mice were anesthetized with zolletil (Virbact)/xylazine (Bayer), and a total of 10⁵ 4T1 cells or 2 × 10⁵ MDA-MB-231 cells (suspended in 100 μl ice cold PBS) were injected into the right inguinal mammary fat pad of BALB/c or NOD-SCID mice, respectively. Tumor volumes were calculated as 0.5 × length × width². Necropsy was performed on 16 (Balb/c) or 28 days (NOD-SCID) after tumor cell implantation. Resected tumor mass was dissected into thin slices and incubated for 30 min with 2 mg/ml collagenase IV (Roche Applied Science) and Dnase 1 (Sigma Aldrich) at 37 °C. Single cell suspensions were washed three times and fixed with 3% formalin followed by methanol until examination. Tunnel assay was performed using FlowTACS flow cytometry apoptosis detection kit (R&D Systems) according to the manufacturer’s instructions. TAMs were first gated with anti-mouse F4/80 PE (ebioscience) and anti-mouse CD45 Qdot650 (ebioscience) double positive population, and the FITC-positive apoptotic cells were gated and analyzed.

Statistical Analysis—Statistical analysis was performed using the unpaired Student’s t test with GraphPad Prism (version 5). Data are presented as means ± S.D.

RESULTS

Reanalysis of Published MDA-MB-231 versus MCF-7 Microarray Results—To identify differences in the regulation of macrophages between malignant and benign tumors, we selected the two human breast cancer cell lines MDA-MB-231 (human malignant breast adenocarcinoma) and MCF-7 (human benign breast adenocarcinoma). We reanalyzed previously published microarray data of MDA-MB-231 and MCF-7 (GSM253207, GSM253208, GSM307014, GSM307015, GSM388187, GSM388191) in terms of differences in gene expression profiles between MDA-MB-231 and MCF-7. We identified 16 genes whose expression was more than 25-fold higher in MDA-MB-231 cells (Table 1). Given that several proteins can influence macrophage behavior, we selected PDGF-C based on its known paracrine effects. Next, we determined whether various cancer cell lines, including MDA-MB-231, expressed PDGF-C.

PDGF-C Expression in Cancer Cell Lines—To assess the production and secretion of PDGF-C by various cancer cell lines, we collected culture supernatants from MDA-MB-231, MCF-7, MeWo, A549, and HT-29 cells after 48 h of incubation in serum-free medium. The PDGF-C contents of the supernatants were assayed by Western blotting under reducing and non-reducing conditions (Fig. 1). Reducing proteins of both the full-length monomer of PDGF-C at 48 kDa and the processed GDF monomer (GDF-M) expression at 17 kDa were detected in only the MDA-MB-231 culture supernatant (Fig. 1A). Western blotting under non-reducing conditions confirmed the full-length dimer of PDGF-C (85 kDa), full-length monomer (48 kDa), and...
the GFD dimer (GFD-D) (26 kDa) (Fig. 1B). These results indicate that MDA-MB-231 breast cancer cells process PDGF-C into the biologically active GFD dimer form under these culture conditions. MCF-7, and the other cell lines secreted a very low level of the full-length dimer (Fig. 1B), highlighting that the metastatic breast cancer cell line MDA231 produces PDGF-C. The PDGF-C gene expression levels in the cancer cell lines were also compared using real-time PCR analysis. As expected, the PDGF-C gene expression levels in MDA-MB-231 were higher than those in the other cancer cell lines used (Fig. 1C). Furthermore, concentrated MDA-MB-231 culture medium induced PDGF-α phosphorylation in THP-1 macrophages (Fig. 1D). Collectively, these data suggest that the malignant breast cancer cell line MDA-MB-231

### TABLE 1

| Gene                              | Gene symbol | Average fold change | p*        |
|-----------------------------------|-------------|---------------------|-----------|
| 1 Dual-specificity phosphatase 6  | Dusp6       | 144                 | 0.00028   |
| 2 Annexin A1                      | Arxa1       | 77                  | 0.02807   |
| 3 Lactate dehydrogenase B         | Ldhb        | 45                  | 0.00100   |
| 4 V-raf murine sarcoma virus-1 oncoprotein homolog F (avian) | Maff | 44                  | 0.01965   |
| 5 Pleckstrin homology-like domain, family A, member 1 | Phlda1 | 42                  | 0.00016   |
| 6 platelet-derived growth factor C | Pdgfc       | 41                  | 0.01342   |
| 7 Coagulation factor II (thrombin) receptor-like 1 | F2rl1 | 29                  | 0.00001   |
| 8 Jagged 1 (Alagille syndrome)     | Jag1        | 28                  | 0.01781   |
| 9 Solute carrier family 16, member 3 (monocarboxylate transporter 4) | Slc16a3 | 28                  | 0.00312   |
| 10 N-cadherin binding protein-like 1 | Stambpl1 | 26                  | 0.00295   |
| 11 Major histocompatibility complex, class I, C | Hla-c | 26                  | 0.00815   |
| 12 Jagged 1 (Alagille syndrome)     | Jag1        | 26                  | 0.01791   |
| 13 Mastermind-like 2 (Drosophila) | Maml2       | 26                  | 0.00298   |
| 14 Metallothionein 1E              | Mtl1e       | 26                  | 0.00015   |
| 15 Sushi-repeat-containing protein, X-linked | Srpx | 25                  | 0.00112   |

* Statistical analysis was performed using Welch’s t test.
produced biologically active PDGF-C that could activate PDGF-Rα on THP-1 macrophages.

**Macrophage PDGF Receptor Expression**—Before investigating PDGF-C-mediated signaling in macrophages, we examined macrophage surface PDGF receptor α and β expression using FACS (Fig. 2). THP-1 cells were differentiated with PMA (100 ng/ml) for 3 days and then were rested for a further 5 days in fresh medium without PMA (Fig. 2A). Increased PDGF receptor α expression along with macrophage differentiation was observed; similar results were also seen in the human peripheral blood mononuclear cell-derived macrophage differentiation process (Fig. 2B). These results indicated that mature human macrophages express surface PDGF-R and, thus, could respond to tumor-derived PDGF-C.

**PDGF-C Phosphorylates PDGF-Rα, Akt, and Bad**—The PDGF receptor is a trans-membrane tyrosine kinase receptor, the tyrosine kinase activity of which is encoded within the intracellular domain of PDGFRs (α and β). Proteolytically activated PDGF-C stimulates dimerization and phosphorylation of PDGFR-α but also activates PDGFR-β by inducing heterodimerization of PDGFR-αβ (10, 13). As expected, recombinant PDGF-C induced phosphorylation of PDGFR-α, starting at 5 min after treatment in THP-1 macrophages (Fig. 3A). We also examined PDGFR-β; however, this receptor showed little phosphorylation after PDGF-C treatment (data not shown). Next, we examined the known PDGFR-mediated PI3K/Akt signaling pathway. PDGF-C phosphorylated Akt at serine 473 at 5 min after treatment (Fig. 3B), and phosphorylation was completely inhibited by the PI3K inhibitor LY294002, indicating PI3K-dependent Akt activation (Fig. 3D). Among the downstream effects of PI3K/Akt, we detected Bad phosphorylation at Ser112 from 10 min after PDGF-C stimulation (Fig. 3C). LY294002 consistently inhibited PDGF-C-mediated Bad phosphorylation (Fig. 3E), suggesting that Bad phosphorylation is dependent on PI3K. Because growth factor activation of the PI3K/Akt signaling pathway culminates in the phosphorylation of Bad and promotes cell survival (32, 33), these results prompted us to evaluate the anti-apoptotic effects of PDGF-C in macrophages.

**Anti-apoptotic Effects of PDGF-C on Human Macrophages**—Next, we determined whether PDGF-C could protect THP-1 macrophages from apoptosis, thus providing some evidence to explain the increased TAM density in breast cancer patients with a poor prognosis. We evaluated the ability of PDGF-C to protect THP-1 macrophages as well as primary human macrophages from STS-induced apoptosis. Macrophages were treated with 200 nM STS for 3 h with or without PDGF-C (100 ng/ml) pretreatment for 24 h (Fig. 4). Macrophage apoptosis was determined by surface annexin V staining using FACS. As expected, PDGF-C protected both THP-1 macrophages and human primary macrophages against STS-induced apoptosis (Fig. 4, A and B). The annexin V-FITC mean fluorescence intensity in the PDGF-C-pretreated cells before STS challenge was almost half that of the STS-only treated cells. Because PDGF-C also reduced the basal apoptotic rate of THP-1 macrophages, we investigated the influence of PDGF-C on the natural death of THP-1 macrophages. After PMA (100 ng/ml) stimulation for 3 days, THP-1 macrophages were further rested for an additional 7 days. The culture media were changed with fresh media every other day. As shown in Fig. 4C, PDGF-C treatment increased the attached cell density at resting day 7. Incubation of THP-1 macrophages with PDGF-C led to a significant increase in cell viability as determined by the MTT assay (Fig. 4D). These results confirmed the

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**FIGURE 2.** PDGF receptor α and β expression in human macrophages. Expression of PDGF receptor α and PDGF receptor β were assayed by flow cytometry. A, THP-1 cells were differentiated with 100 ng/ml PMA for 3 days, and then were further rested for 5 days. Resting day 3 and 5 macrophages were stained with PDGF receptor antibody and examined using FACS. B, Human blood monocytes were differentiated with 50 ng/ml M-CSF for 7 days. Differentiation day 5 and 7 macrophages were stained with PDGF receptor antibody and examined using FACS. Results are representative of three independent experiments.
protective effect of PDGF-C on THP-1 macrophages from apoptosis, resulting in enhanced survival.

**PDGF-C Inhibits Caspase Activation in THP-1 Macrophages during STS-induced Apoptosis**—Caspase activation and cleavage of PARP-1 play critical roles in the apoptosis process (34, 35). Hence, activation of caspase -3, -7, -8, and -9 and PARP cleavage were examined by Western blotting (Fig. 5). Caspase-3, -7, -8, and -9 were activated in THP-1 macrophages exposed to STS; however, PDGF-C pretreatment significantly reduced caspase activation. STS-induced caspase-3 activation was almost completely
inhibited by PDGF-C, whereas caspase-8 and caspase-9 activation was inhibited partially (Fig. 5). PARP cleavage was protected by PDGF-C pretreatment for 24 h prior to STS challenge. Notably, 24 h of PDGF-C pretreatment reduced basal caspase-3, -7, -8, and -9 activation and PARP cleavage rates in THP-1 macrophages. Collectively, these results show that PDGF-C enhances macrophage survival by inhibiting early apoptotic events.

Neutralization of PDGF-C Inhibits the Anti-apoptotic Effects of MDA-MB-231-conditioned Medium—To determine whether PDGF-C secreted from MDA-MB-231 cells can inhibit apoptosis, we investigated the anti-apoptotic effects of MDA-MB-231-conditioned medium with or without PDGF-C neutralizing antibody on THP-1 macrophages by annexin V staining. As expected, apoptosis induced by STS was prevented by preincubation with MDA-MB-231-conditioned medium for 24 h (Fig. 6, A and B). PDGF-C neutralizing antibody again inhibited the anti-apoptotic effects of MDA-MB-231-conditioned medium, as evidenced by the increased annexin V-FITC mean fluorescence intensity values (Fig. 6B). Thus, our results provide evidence for the anti-apoptotic effects of MDA-MB-231-conditioned medium mediated by PDGF-C.

PDGF-C Knockdown in Breast Cancer Cells Could Not Prevent Macrophage Apoptosis in the Tumor Microenvironment—Finally, we examined the effects of tumor cell-derived PDGF-C on macrophage apoptosis in the tumor environment using a mouse breast cancer model. We selected a 4T1 orthotopic syngeneic mouse model, because it can provide the most natural tumor immunological microenvironment. We established stable PDGF-C knockdown in 4T1 and MDA-MB-231 cell lines using shRNA viral vector and confirmed down-regulation of PDGF-C mRNA expression compared with expression in WT cells using real-time PCR (Fig. 7A). Cells were implanted into the mammary fat pad of female Balb/c mice or NOD-SCID mice, respectively. Surprisingly, the tumor mass size was persistently down-regulated in the 4T1 knockdown group (Fig.
B), although no detectable difference in tumor cell growth rate was found compared with WT cells in vitro (data not shown). To examine apoptosis in TAMs, TUNEL staining was performed in single-cell isolates from the tumor mass, and additional F4/80 and CD45 staining was conducted to gate the macrophage population (Fig. 7C). Consistent with our in vitro data, macrophages from PDGF-C knockdown cell-implanted mouse tumors showed higher apoptotic rates compared with the control (Fig. 7D). We also confirmed similar results in the MDA-MB-231 xenograft model using NOD-SCID mice (Fig. 7D). Collectively, we showed that aggressive breast cancer cells prevent apoptosis of macrophages through PDGF-C secretion, and this interaction could affect tumor progression.

**DISCUSSION**

Although PDGF-C is known to be an important regulator of cell growth and apoptosis (36–39), the present study is the first to demonstrate its anti-apoptotic effects on macrophages and the underlying mechanisms of action. Because macrophages constitute the major cell population present in tumors and contribute to disease progression (16, 20, 22), our findings provide important insights into malignant cell-derived PDGF-C production and macrophage survival in the tumor microenvironment.

TAMs produce a unique tumor microenvironment that can modify the neoplastic properties of both tumor and surrounding stromal cells. Thus, the relationship between TAMs and tumors has been extensively researched in the past. Based on accumulated data, it is generally thought that TAMs function mostly to promote tumor growth, metastasis, and angiogenesis (16, 23, 40). Along with its supposed tumor-promoting roles, the extent of TAM infiltration has been studied as a prognostic factor (40–42). For example, in some human tumors, i.e., breast, thyroid, and esophageal cancers, the extent of macrophage infiltration was correlated with clinical aggressiveness (43–45). These findings have led to an evaluation of macrophage targeting in a tumor setting (46–48). The
fundamental principles of TAM-targeted anti-tumor approaches are based on inhibiting macrophage recruitment (49–51), suppressing TAM survival (52–54), enhancing the anti-tumor activity of TAMs (55–57), and blocking their protumor activity (58–60). Targeting TAMs is thus an important and efficient strategy for cancer therapy. However, the effectiveness of these approaches as a potential therapy should be further explored, as protumoral TAMs could be depleted, whereas anti-tumoral TAMs may not be affected.

Cytokines, chemokines, growth factors, and proteolytic enzymes from tumor cells and stromal cells are known to facilitate the recruitment of macrophages to tumor tissues (40, 42, 61). For example, overexpression of the chemoattractants CCL2, CCL5, IL-6, and VEGF was correlated with increased macrophage infiltration and poor prognosis in human cancers (62–65); this finding has been a focus of clinical trials of anti-tumor drugs that have the potential to target TAMs (46). Actually, we found that up to 10–20% of cells in the tumor mass comprised mature macrophages in mouse breast cancer models (Fig. 7). Intriguingly, there were fewer infiltrated macrophages in the PDGF-C knockdown cell-derived tumor mass (data not shown), a finding that is consistent with a reduced tumor growth rate as well as an up-regulated macrophage apoptotic rate. Because the tumor microenvironment might be insufficient for appropriate macrophage survival caused by hypoxia or nutrient competition with actively proliferating cells, it can be assumed that infiltrated macrophages easily undergo apoptosis. However, in the current study, we revealed the possible survival mechanisms of macrophages in relation to abundant PDGF-C from aggressive breast cancer cells.

PDGFs are classified as members of the superfamily of growth factors (10, 39, 66, 67). Because PDGFs show high sequence identity with VEGFs, they are often referred to collectively as the PDGF/VEGF family (68). PDGFs are important in connective tissue growth and survival (10, 12, 69). Recently, the role of PDGFs in lymphocyte survival, via an autocrine regulating pathway, was investigated (70). Comparison of VEGFs and PDGFs and their receptor properties suggests that PDGFs may have an impact on TAM apoptosis. Based on this possibility, we hypothesized that PDGF-C could have anti-apoptotic effects on macrophages. Interestingly, the microarray data from up-regulated PDGF-C in MDA-MB-231 compared with MCF-7 were correlated with the tissue microarray analysis results of 216 patients with invasive malignant breast cancer (3). However, no experimental evidence to date has demonstrated a direct effect of PDGF-C on macrophages. In the present study, we demonstrated that the MDA-MB-231 human malignant breast adenocarcinoma cell line produced biologically active PDGF-C that could activate PDGFR-α on THP-1 macrophages. Concentrated MDA-MB-231 culture medium failed to inhibit STS-induced apoptosis in the presence of PDGF-C neutralizing antibodies (Fig. 6). Therefore, PDGF-C might represent a potential anti-cancer therapy target to modulate macrophage survival rates.

The PI3K/Akt signaling pathway plays a critical role in various cellular events such as apoptosis, cell cycle progression, and transcriptional regulation (71–73). The ability of Akt to prevent apoptosis in cells through phosphorylation and inhibition of proapoptotic mediators such as Bad and caspase-9 has been well documented (74). Hu et al. (75) demonstrated that activation of the PI3K pathway by PDGFRs promotes actin reorganization, cell movement, cell growth, and inhibition of apoptosis. PI3K signaling by PDGFs both directly and indirectly regulates the apoptotic pathway through the action of effectors, including serine/threonine kinases such as Akt/PKB (76, 77). Akt activity is important for cell survival through regulation of multiple target pathways by phosphorylation of critical proteins. For example, phosphorylation of Bad at Ser<sup>112</sup> by Akt induces a conformational change that blocks the ability of Bad to interact with anti-apoptotic Bcl-2 proteins (78). These free anti-apoptotic Bcl-2 proteins could then inhibit Bax-triggered apoptosis by maintaining the integrity of the outer mitochondrial membrane (78–80). Mitochondria play important roles in the regulation and transmission of apoptotic signals, which are regulated by the balance among Bcl-2 family members; therefore, phosphorylation of Bad at Ser<sup>112</sup> promotes cell survival (32, 74, 81). In the present study, we showed that the PDGF-C-mediated anti-apoptotic signaling pathway was mediated via PI3K/Akt activation and proapoptotic Bad phosphorylation at Ser<sup>112</sup>, culminating in Bad inactivation of THP-1 macrophages. Subsequently, phosphorylated Bad inhibited caspase activation and PARP cleavage (Fig. 4). However, Weisser et al. (82) reported that PI3K could activate the M2 skewing of macrophages through increasing STAT6 activity. Therefore, PI3K activation by PDGF-C led to an increase in immune-suppressive TAM via an anti-apoptotic mechanism.

Taken together, our results demonstrate the anti-apoptotic effect of PDGF-C on macrophages and clarify the signaling pathway of PDGF-C-mediated receptor α phosphorylation, followed by PI3K/Akt activation and Bad inactivation. These events, in turn, lead to inhibition of caspase activation and PARP cleavage (Fig. 8). These findings support a therapeutic role for tumor-derived PDGF-C that is mediated by its promotion of TAM survival.

REFERENCES

1. Heldin, C. H., Ostman, A., and Rönnstrand, L. (1998) Signal transduction via platelet-derived growth factor receptors. Biochim. Biophys. Acta 1378,
Effect of PDGF-C on Macrophage Survival

Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 23, 549–555

Pollard, J. W. (2004) Tumour-educated macrophages promote tumour progression and metastasis. Nat. Rev. Cancer 4, 71–78

Leek, R. D., Hunt, N. C., Landers, R. J., Lewis, C. E., Royds, J. A., and Harris, A. L. (2000) Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. J. Pathol. 190, 430–436

Tsutsui, S., Yasuda, K., Suzuki, K., Tahara, K., Higashi, H., and Era, S. (2005) Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. Oncol. Rep. 14, 425–431

Kawata, M., Koinuma, D., Ogami, T., Umezawa, K., Iwata, C., Watabe, T., and Miyazono, K. (2012) TGF-β-induced epithelial-mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells. J. Biochem. 151, 205–216

Katsuno, Y., Lamouille, S., and Derynk, R. (2013) TGF-β signaling and epithelial-mesenchymal transition in cancer progression. Curr. Opin. Oncol. 25, 76–84

Marconi, C., Bianchini, F., Mannini, A., Mugnai, G., Ruggieri, S., and Calorini, L. (2008) Tumoral and macrophage uPAR and MPP-9 contribute to the invasiveness of B16 murine melanoma cells. Clin. Exp. Metastasis 25, 225–231

Tejada, M. L., Yu, L., Dong, J., Jung, K., Meng, G., Peale, F. V., Frantz, G. D., Hall, L., Liang, X., Gerber, H. P., and Ferrari, N. (2006) Tumor-driven paracrine platelet-derived growth factor receptor α signaling is a key determinant of stromal cell recruitment in a model of human lung carcinoma. Clin. Cancer Res. 12, 2676–2688

Lokker, N. A., Sullivan, C. M., Holbenbach, S. J., Israel, M. A., and Giese, N. A. (2002) Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer Res. 62, 3729–3735

Andrae, J., Molander, C., Smits, A., Funa, K., and Nistér, M. (2002) Platelet-derived growth factor-B and -C and active α-receptors in murine melanoma cells. Biochem. Biophys. Res. Commun. 99, 604–611

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotth, Y., and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231–241

Danial, N. N. (2008) BAD: undertaker by night, candyman by day. Oncogene 27, 553–70

Skommer, J., Wlodkowic, D., and Deptala, A. (2007) BAD: undertaker by night, candyman by day. Oncogene 27, 553–70

Romashkova, J. A., and Makarov, S. S. (1999) NF-κB is a target of AKT in anti-apoptotic PDGF signaling. Nature 401, 86–90

Vantler, M., Caglayan, E., Zimmermann, W. H., Bäumer, A. T., and Rosenkranz, S. (2005) Systematic evaluation of anti-apoptotic growth factor signaling in vascular smooth muscle cells. Only phosphatidylinositol 3'-kinase is important. J. Biol. Chem. 280, 14168–14176

McDonald, N. Q., and Hendrickson, W. A. (1993) A structural superfamily of growth factor receptors containing a cystine knot motif. Cell 73, 421–424

Joyce, J. A., and Pollard, J. W. (2009) Microenvironmental regulation of metastasis. Nat. Rev. Cancer 9, 239–252

Smith, H. A., and Kang, Y. (2013) The metastasis-promoting roles of tumor-associated immune cells. J. Mol. Med. 91, 411–429

Gocheva, V., Wang, H. W., Gadea, B. B., Shree, T., Hunter, K. E., Garfall, A. L., Berman, T., and Joyce, J. A. (2010) IL-4 induces cathespin protease activity in tumor-associated macrophages to promote cancer growth and invasion. Genes Dev. 24, 241–255

Azenshtein, E., Luboshits, G., Shina, S., Neumark, E., Shahbazian, D., Weil, M., Wigler, N., Keydar, I., and Ben-Baruch, A. (2002) The CC chemokine
RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Cancer Res. 62, 1093–1102

Bingle, L., Brown, N. I., and Lewis, C. E. (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J. Pathol. 196, 254–265

Wei, Q., Fang, W., Ye, L., Shen, L., Zhang, X., Fei, X., Chen, X., Li, X., Xiao, J., and Ning, G. (2012) Density of tumor associated macrophage correlates with lymph node metastasis in papillary thyroid carcinoma. Thyroid. 22, 905–910

Tang, X., Mo, C., Wang, Y., Wei, D., and Xiao, H. (2013) Anti-tumour strategies aiming to target tumour-associated macrophages. Immunology 138, 93–104

Yue, Z. Q., Liu, Y. P., Ruan, J. S., Zhou, L., and Lu, Y. (2012) Topical imidazoquinoline therapy of cutaneous squamous cell carcinoma polarizes lymphoid and tumor-associated macrophages. Immunology 138, 93–104

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Thompson, R., Schioppa, T., Nemeth, J., Vermeulen, J., Singh, N., Avril, N., Cummings, I., Rexhepi, E., Jirström, K., Gallagher, W. M., Brennan, D. J., McNeish, I. A., and Balkwill, F. R. (2011) Interleukin-6 as a therapeutic target in human ovarian cancer. Clin. Cancer Res. 17, 6083–6096

Sitohy, B., Nagy, J. A., and Dvorak, H. F. (2012) Anti-VEGF/VEGFR therapy for cancer: reassessing the target. Cancer Res. 72, 1909–1914

Soria, G., and Ben-Baruch, A. (2008) The inflammatory chemokines CCL2 and CCL5 in breast cancer. Cancer Lett. 267, 271–285

Roca, H., Varsos, Z. S., Sud, S., Craig, M. J., Ying, C., and Pienta, K. J. (2009) CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization. J. Biol. Chem. 284, 34342–34354

Sun, P. D., and Davies, D. R. (1995) The cytokine-knot growth-factor superfamily. Ann. Rev. Biophys. Biomol. Struct. 24, 269–291

Vitt, U. A., Hsu, S. Y., and Haue, A. J. (2001) Evolution and classification of cytokine-knot-containing hormones and related extracellular signaling molecules. Mol. Endocrinol. 15, 681–694

Reigstad, L. J., Varhaug, J. E., and Lillehaug, J. R. (2005) Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. FEBS J. 272, 5732–5741

Ding, H., Wu, X., Kim, I., Tam, P. P., Koh, G. Y., and Nagy, A. (2000) The mouse Pdgfc gene: dynamic expression in embryonic tissues during organogenesis. Mech. Dev. 96, 209–213

Yang, J., Liu, X., Nyland, S. B., Zhang, R., Ryland, L. K., Broeg, K., Baab, K. T., Jarbadan, N. R., Irby, R., and Loughran, T. P., Jr. (2010) Platelet-derived growth factor mediates survival of leukaemic large granular lymphocytes via an autocrine regulatory pathway. Blood 115, 51–60

Lu, X., Masic, A., Li, Y., Shin, Y., Liu, Q., and Zhou, Y. (2010) The PI3K/Akt pathway inhibits influenza A virus-induced Bax-mediated apoptosis by negatively regulating the JNK pathway via ASK1. J. Gen. Virol. 91, 1439–1449

Dey, J. H., Bianchi, F., Voshol, J., Bentenf, D., Oakeley, E. J., and Hynes, N. E. (2010) Targeting fibroblast growth factor receptors blocks PI3K/AKT signaling, induces apoptosis, and impairs mammary tumor outgrowth and metastasis. Cancer Res. 70, 4151–4162

Jeong, S. J., Dasgupta, A., Jung, K. J., Um, J. H., Burke, A., Park, H. U., and Brady, J. N. (2008) PI3K/AKT inhibition induces caspase-dependent apoptosis in HTLV-1-transformed cells. Virology 370, 264–272

Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cellular survival: a play in three acts. Genes Dev. 13, 2905–2927

Hu, Q., Klippel, A., Muslin, A. J., Fantl, W. J., and Williams, L. T. (1995) Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. Science 268, 100–102

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81, 727–736

Duronio, V. (2008) The life of a cell: apoptosis regulation by the PI3K/Akt pathway. Biochem. J. 415, 333–344

Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAX by BH3 domain phosphorylation. Mol. Cell 6, 41–51

Willis, S. N., Fischer, J. I., Kaufmann, T., van Delft, M. F., Chen, L., Czbator, P. E., Ierino, H., Lee, E. F., Fairlie, W. D., Bouillet, P., Strasser, A., Kluck, R. M., Adams, J. M., and Huang, D. C. (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 315, 856–859

Chipuk, J. E., Bouchier-Hayes, L., and Green, D. R. (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ. 13, 1396–1402

Quan, J. H., Cha, G. H., Zhou, W., Chu, J. Q., Nishikawa, Y., and Lee, Y. H. (2013) Involvement of PI3 kinase/Akt-dependent Bad phosphorylation in Toxoplasma gondii-mediated inhibition of host cell apoptosis. Exp. Parasitol. 133, 462–471

Weisser, S. B., McLarren, K. W., Vologmaier, N., van Netten-Thomas, C. J., Antov, A., Flavell, R. A., and Sly, L. M. (2011) Alternative activation of macrophages by IL-4 requires SHIP degradation. Eur. J. Immunol. 41, 1742–1753