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Lymphangiogenesis Linked to VEGF-C from Tumor-Associated Macrophages: Accomplices to Metastasis by Cutaneous Squamous Cell Carcinoma?

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During wound healing, dermal macrophages secrete lymphangiogenic vascular endothelial growth factor (VEGF)-C, and lymphatic vessels transport cytokines and cells to draining lymph nodes. In this issue, Moussai *et al.* show that macrophages in peritumoral nonlesional skin near squamous cell carcinoma secrete prolymphangiogenic VEGF-C. Their study suggests how tumor-associated macrophages and neolymphatic vessels may coordinate metastasis starting early in cutaneous squamous cell carcinoma.

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Roles for macrophage and lymphatic endothelial cells near squamous cell carcinoma

The primary functions of macrophages were first characterized in settings of classical inflammation in which they exert antimicrobial activity, serve as antigen-presenting cells required for the adaptive immune response, and promote healing by tissue remodeling at sites of injury. In contrast, tumor-associated macrophages (TAMs) are less inflammatory and contribute to tissue remodeling by promoting the proliferation and migration of endothelial cells (ECs) that lead to growth of new lymphatic vessels (lymphangiogenesis). The presence of TAMs generally correlates with a poor prognosis in most human cancers. Importantly, whereas lymphangiogenesis observed in many aggressive cancers correlates with metastasis to regional lymph nodes (e.g., in cutaneous melanoma and in head/neck and oral squamous cell carcinoma (SCC)), the presence and characterization of prolymphangiogenic TAMs in cutaneous SCC has not been previously described.

In this issue, Moussai *et al.* describe $CD68^+/CD163^+$ TAMs in peritumoral nonlesional skin (PTNL) of stage I cutaneous SCC as a source of vascular endothelial growth factor (VEGF)-C that correlates with an increase in lymphatic vessel density (LVD). These data showing lymphangiogenic VEGF-C produced by a defined subpopulation of TAMs raise a number of interesting questions about how macrophage-driven lymphangiogenesis may promote metastasis in SCC. Furthermore, these findings invite investigation of molecules expressed by lymphatic endothelial cells (LECs), or at recruitment of prolymphangiogenic TAMs, as potential therapeutic targets for preventing life-threatening metastases to regional draining lymph nodes.

Trophic macrophages are recruited by wounds and tumors

Macrophages are bone marrow–derived cells that initially circulate as monocytes and subsequently differentiate into tissue-resident macrophages. Resident macrophages support tissues by phagocytosing apoptotic cells, and they become trophic when activated, secreting growth, angiogenic, and lymphangiogenic factors needed for tissue remodeling. Wounding of tissues triggers an acute inflammatory response, characterized by the production of numerous cytokines and chemokines, which recruit and differentiate additional circulating monocytes into macrophages. Wound-associated macrophages have been proposed to coordinate new tissue formation and remodeling. More specifically, macrophages have been found to regulate vasculogenesis in wound healing. Whereas the specific

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Clinical Implications

- Responses of dermal macrophage and lymphatic endothelial cells may serve as prognostic indicators for cutaneous squamous cell carcinomas.
- Investigation of molecules expressed by lymphatic endothelial cells or prolymphangiogenic tumor-associated macrophages may reveal potential therapeutic targets for treatment of early skin cancers.
- Investigation of such molecules may also lead to early detection of aggressive disease or the prevention of life-threatening metastases.

roles of wound-associated macrophages are incompletely understood, TAMs appear to perform similar functions in the tumor environment.

A variety of tumors that recruit macrophages to tumors have been found to produce cytokines in a manner similar to that of wounds (Condeelis and Pollard, 2006). VEGF-A is proinflammatory as well as proangiogenic and prolymphangiogenic, and its levels are elevated in cutaneous SCC. At sufficiently high levels, the leukocyte adhesion molecule ICAM-1 is inducible by VEGF, which may synergize with tumor necrosis factor (also present in the SSC microenvironment) to further induce ICAM-1 and other leukocyte adhesion molecules on the lumenal surface of dermal postcapillary venules. Efficient recruitment also requires that surface chemokines on blood endothelial cells (BECs) engage cognate receptors on circulating monocytes. The inflammatory cytokine IL-8, noted as upregulated in SCC by Moussai *et al*. (2011), may provide for TAM recruitment because firm adhesion of monocytes to vascular endothelium is IL-8 dependent (Gerszten *et al*., 1999).

Tumor-associated macrophages promote tumorigenesis

Whereas the role of these TAMs has yet to be clearly characterized, a majority (>80%) of clinical studies correlate an increased number of TAMs with a worse prognosis (Pollard, 2004). Several *in vivo* studies have supported these clinical observations: mice deficient in colony-stimulating factor-1 (CSF-1) and thus deficient in macrophages, when crossed with mice expressing a breast epithelium-specific oncogene, exhibit delayed tumor progression and nearly no metastases (Lin *et al*., 2001). Local transgenic expression of CSF-1 in the breasts of these CSF-1-deficient mice results in a rate of metastasis equivalent to that of wild-type mice, suggesting that macrophages are necessary for metastasis.

Macrophages can be found in most tissues of the body and as such serve a broad variety of functions. Classification of macrophage subtypes has been attempted according to functional and transcriptional differences. M1, or "classically activated," macrophages require priming by the T helper type 1 (Th1)-type cytokines IFN-γ, IL-12, and IL-23 or by microbial products such as lipopolysaccharide. M1 macrophages are best characterized for their cell-mediated antimicrobial functions. M2, or "alternatively activated," macrophages are primed by the Th2-type cytokines IL-4 and IL-13 and have functions that include humoral-mediated antimicrobial functions as well as tissue repair and fibrosis. The characterization of M1 and M2 macrophages defines extremes of a spectrum of phenotypes. Whereas the TAM transcriptional program is unique, it reveals an M2-like phenotype with an increase in expression of the immunosuppressive cytokine IL-10, lymphangiogenic and angiogenic growth factors, metalloproteases, and IFN-inducible cytokines (Pollard, 2009). In cervical SCC, where LVD is increased in the peritumoral stroma, TAMs that are recruited to the tumor as monocytes are subsequently "educated" by the tumor environment, and the same process may occur with cutaneous SCC (referenced in Moussai *et al*., 2011). But whereas the factors required for priming monocytes to become M1 and M2 macrophages have been well characterized, the factors that prime recruited monocytes to become TAMs have yet to be identified.

Tumor-associated macrophages express VEGF-C

It has been well established that macrophages have a significant biosynthetic capacity for secreted proteins, including angiogenic factors. Elevated levels of VEGF-C expression and LVD have been positively correlated with metastasis in breast, colon, lung, and prostate cancers. Certainly in human melanoma increased LVD has emerged as a rational basis for flagging high risk for nodal metastasis and decreased survival (Dadras *et al*., 2005). Germane to the current study, VEGF-C expression has been associated with tumor progression in SCCs of the head and neck and cervix. Moussai *et al*. demonstrate for the first time that cutaneous SCC, like cervical SCC, has increased LVD as compared with normal skin and that TAMs are the stromal cell type responsible for the production of VEGF-C. The factors within the tumor environment that induce the production of VEGF-C are the subject of intense investigation. Whether these findings correlate with a prognosis indicating increased propensity for nodal metastasis in cutaneous SCC has yet to be determined.

Structure and function of normal and tumor-associated lymphatic vessels

The physiologic role for tumor-associated lymphangiogenesis may recapitulate programs of wound healing and/or development. First described as "milky veins" in the seventeenth century, lymphatic vessels provide unidirectional transport of fluid, macromolecules, and antigen-presenting cells to draining lymph nodes. The lymphatic system sprouts from the existing blood microvasculature during embryonic development in response to VEGF-C to form primitive lymph sacs and, in an orderly progression, expresses genes specifying lymphatic identity (early markers are Prox1, VEGFR-3, and podoplanin). Later, maturation of the lymphatic vasculature involves angiopoietin 2 and new gene expression (of ephrinB2, neuropilin2, and transcription factors such as Foxc2). It is still unclear how lymphangiogenesis near SCC tumors may recapitulate the carefully orchestrated developmental program of lymphangiogenesis.

The mechanism(s) by which tumor cells gain entry to lymphatics is not well described. Compared with blood vessels, lymphatics are thin walled and lack a continuous basement membrane. Recent evidence shows VE-cadherin and tight junction proteins (occludin, claudin-5, JAM-A, ESAM) expressed at the initial openings of lymphatics (Baluk *et al.*, 2007). Described as buttons designed to open and close for leukocyte entry, to the extent that lymphatic entry by cells is a selective process, such structures may be relevant for regulating tumor metastasis. Intraendothelial channels found in normal and tumor-associated lymphatic vessels support an alternative, transendothelial modality for tumor cell intravasation that is junction independent. VEGF-C stimulates dilation of preexisting lymphatics, corresponding to an increase in LN-directed flow, so it is also possible that lymphangiogenesis may promote metastasis simply by increasing the rate of transport and/or amount of lymphatic surface available for contact with malignant cells.

Targeting the VEGF-C pathway to impede metastasis

VEGF-C signals through the tyrosine kinase receptor VEGFR-3 (Flt-4) that in normal healthy adult skin is restricted to the lymphatic vasculature. Early in development, continuous VEGFR-3 signaling ensures survival of lymphatic EC, but this dependency wanes in postnatal development. Therefore, VEGFR-3-inhibitory therapies may spare normal lymphatic cells while selectively interfering with the formation of new peritumoral lymphatic vessels. Cancer investigators have pursued an assortment of strategies by targeting VEGFR-3 with neutralizing antibodies, decoy VEGFR-3 fusion proteins, and siRNA approaches. Still to be gauged, however, is the extent to which dermal lymphatic vessels formed during development contribute or whether tumoral lymphangiogenesis is absolutely required for nodal metastasis to occur in human cutaneous SCC.

Only in the past decade have antibody tools for differentiating LECs become available, coinciding with comprehensive studies comparing differential gene expression by purified human blood and lymphatic microvascular ECs. Moussai *et al.* (2011) used a database of genes enriched in cultured LECs over cultured BECs (see Supplemental Table III in Petrova *et al.*, 2002) for gene set enrichment analysis on cDNA transcripts derived from the PTNL of cutaneous SCC. Interestingly, more so than normal skin or intratumoral SSC, nonlesional skin adjacent to tumors was enriched for gene transcripts found highly expressed by differentiated LECs. Moussai *et al.* (2011) also used quantitative real-time reverse transcription–PCR to show that neuropilin-2 (NRP-2), a VEGFR-3 coreceptor for VEGF-C, is elevated in PTNL regions surrounding SSC tumors. NRP-2 was originally found to be important for neuronal axon guidance but has more recently been implicated in regulating tumor lymphangiogenesis and nodal metastases. Specifically, Caunt *et al.* (2008) designed a monoclonal antibody targeting the VEGF-C-binding domain of NRP-2 that reduced LVD near tumors (formed by subcutaneously implanting a murine mammary carcinoma line) without harming normal lymphatics and that significantly delayed metastasis of carcinoma cells to the primary lymph node. Collectively, these data support a role for NRP-2 in controlling lymphangiogenesis and tumor cell migration that deserves further attention with respect to invasive cutaneous SCC.

Conclusion

These observations emphasize that responses of dermal macrophage and LECs may serve as prognostic indicators during early stage I disease and may provide clues toward reaching a deeper understanding of how cutaneous SCC metastasizes. Further investigation of molecules expressed by LECs or pro-lymphangiogenic TAMs as potential therapeutic targets for early detection of aggressive disease or for preventing life-threatening metastases to regional draining lymph nodes is needed.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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