Macrophage infiltration predicts a poor prognosis for the human Ewing sarcoma

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INTRODUCTION:
Ewing sarcoma / primitive neuroectodermal tumor (EWS) is a small round-cell tumor type that typically develops in the bones of children and young adults, and associated with the most unfavorable prognosis of all primary musculoskeletal tumors. Recent studies have highlighted the importance of tumor stroma. Interactions between stromal cells and tumor cells are essential for tumor malignancy including angiogenesis. Among stromal cells, tumor associated macrophages (TAMs) are known to promote tumor angiogenesis, invasion and metastasis in various tumors. TAMs accumulation has a significant correlation with microvascular density (MVD) or tumor progression in glioblastoma and melanoma, and is associated with poor prognosis in breast, prostate, bladder and cervical cancers. Currently, little is known about the role of TAMs in EWS. In this study, TAMs were isolated from mouse EWS xenograft, and the characteristics of these cells were investigated. We also sought to determine the prognostic significance of TAMs in EWS.

MATERIALS AND METHODS:
Tumor xenografts were established in nude mice by subcutaneous inoculation of EWS cell line, RD-ES or TC-71. For macrophage isolation, tumor xenografts or liver/spleen were dissected, treated with collagenase and DNase, and magnetically isolated using CD11b magnetic beads. Liver and spleen were used as a source of control monocytes (CoMs). Cytokine production by TAMs was examined using Luminex multiplex assay. Vascular endothelial growth factor (VEGF) production was examined using ELISA. In vivo macrophage depletion model was performed by administering liposome-encapsulated clodronate (C2MDP-Lip) or PBS-Lip to mice intravenously. To analyze macrophage infiltration and tumor vasculature, immunohistochemistry was performed with clinical samples of 41 cases of EWS, using anti-CD68 and anti-CD31 antibodies, respectively.

RESULTS:
Immunostaining revealed a number of F4/80-positive macrophages among the homogeneous small and round tumor cells in both RD-ES and TC-71 xenografts. Flow cytometry analysis demonstrated that approximately 90% of the isolated cells were positive for both F4/80 and CD11b, suggesting that these cells could be used for further experiments. Expression of various cytokines and chemokines by TAMs was examined by using the Luminex multiplex assay system. Expression levels of factors known to stimulate monocyte chemotaxis, including IL-6, MCP-1, KC, MIP-1, and RANTES, were significantly up-regulated in conditioned medium from TAMs. This data indicates that TAMs in EWS are "activated" macrophages that secrete a number of proinflammatory cytokines. Next investigated were the potential mechanisms underlying recruitment of macrophages to EWS. A cytokine multiplex assay revealed that all six EWS cell lines secreted VEGF, which can induce migration of monocyte cells. In addition, increased VEGF secretion was observed when the RD-ES or TC-71 cells were stimulated with conditioned medium from TAMs (Figure 1). These results demonstrated that recruitment of TAMs to EWS depends, at least in part, on EWS-derived VEGF, the secretion of which is up-regulated in the presence of TAMs. To investigate the involvement of TAMs in development of EWS, C2MDP-Lip was used to decrease macrophages in mouse EWS xenografts. Compared with PBS-Lip, C2 MD Lip significantly inhibited development of xenografts.

Next we examined whether the infiltrating macrophages were associated with clinical outcome in EWS patients. A higher extent of CD68+ macrophage infiltration (p<0.05, log-lank test) and greater MVD (p<0.05, log-lank test) were significantly associated with poorer overall survival (Figure 2). In addition, elevated C-reactive protein (CRP) was also associated with poor prognosis (p<0.05). A higher macrophage infiltration rate was also significantly associated with higher MVD and elevated CRP.

DISCUSSION:
Through production of growth factor and cytokines, TAMs have a key role in angiogenesis in various tumors and associate with poor clinical outcomes. In this study, we demonstrated that infiltrating TAMs associated with angiogenesis and inflammation during EWS development, which suggests that TAMs could be used as a prognostic factor for EWS. TAMs accumulation is mediated by VEGF secretion from EWS, and is further enhanced by various cytokines and chemokines released from the TAMs themselves, resulting in an inflammatory reaction. TAMs stimulate tumor angiogenesis by enhancing VEGF production from tumor cells, resulting in a poorer prognosis (Figure 3).

In conclusion, the present study revealed a significant association between macrophage infiltration and clinical outcome in EWS. TAMs and the various factors that they produce may provide new therapeutic targets for EWS.

Figure 1. Luminex assay screened for cytokine produced by EWS cell lines (left). Quantification of VEGF secretion by EWS cells (RD-ES and TC-71). CM of TAMs stimulated VEGF production of EWS cells (right).

Figure 2. Kaplan-Meier survival curves for all patients based on CD68+ macrophage infiltration (low, ≤30; high, >30) (left) and MVD (low, ≤10; high, >10) (right).

Figure 3. Model for TAM-mediated modulation of EWS microenvironment.

SIGNIFICANCE:
In Ewing sarcoma, TAMs enhance the tumor progression by stimulating inflammation and angiogenesis, and predicts a poor prognosis.

REFERENCES: