Macrophages infiltration associates with poor prognosis in Ewing’s sarcoma

INTRODUCTION:
The Ewing’s sarcoma family of tumors (ESFT) is a small round-cell tumor typically occurring in the bones, rarely in soft tissues, and retained the worth prognosis of all primary musculoskeletal tumors. Although the development of multi-disciplinary therapy with irradiation, surgery and chemotherapy has increased current long-term survival rates, lesser improvements have been seen in patients with metastatic or recurrent disease. Thus far little is known about the mechanisms underlying metastasis and enhanced osteolysis of ESFT.

Tumor associated macrophages (TAMs) are known to promote tumor angiogenesis, invasion and metastasis by producing various cytokines and proteinases in various tumor types. In addition, TAMs are reported to be capable of differentiating into osteoclast, which is an indispensable cell type for the development of bone tumors.

In this study, we investigated whether infiltrating macrophages affected clinical outcome of patients with ESFT. Furthermore, we isolated TAMs from ES xenograft, and examined their biological characteristics concerning cytokine production, cytokine production, angiogenesis and osteoclastogenesis.

MATERIALS AND METHODS:
To analyze macrophage infiltration and tumor vasculature, immunohistochemistry was performed with clinical samples of 41 cases of ESFT, using anti-CD68 and anti-CD31 antibodies, respectively. Tumor xenografts were established in nude mice by subcutaneous inoculation of ESFT cell line, RD. For detecting macrophages, xenografts were immunostained using F4/80 antibody. For macrophage isolation, tumor xenografts or liver/spleen were dissected, treated with collagenase and DNase, and magnetically isolated using CD11b magnetic beads. Cytokine production by TAMs was examined using Luminex multiplex assay.

RESULTS:
We examined whether the number of infiltrating macrophages was associated with clinically poor prognosis of ESFT using Kaplan-Meier survival analysis. The higher levels of CD68 positive macrophages in ESFT (CD68>30) significantly associated with poorer overall survival (Figure 1) \(p<0.05\), log-rank test). As reported previously, higher MIB-1 expression and larger tumor size (>8cm) were associated with poorer prognosis. In addition, enhanced vasculature was also associated with poor prognosis, and significantly correlated with macrophage infiltration \(p<0.05\).

DISCUSSION:
Because infiltrating macrophages significantly correlated with enhanced vasculature in patients with ESFT, we examined how TAMs in ESFT stimulated angiogenesis. VEGF, which is considered to be a key regulator of angiogenesis in ESFT, is mostly derived from tumor cells. Production of VEGF by ESFT cells was significantly up-regulated when ESFT cells were co-cultured with TAMs.

We then examined whether TAMs in ESFT could differentiate into osteoclast. To induce osteoclast differentiation, TAMs or CoMs were incubated with sRANKL and M-CSF for 4 days, and then TRAP activity was visualized. Both TAMs and CoMs differentiated into TRAP-positive giant cells, but much more TRAP-positive giant cells were developed from TAMs. This result indicates that TAMs are more capable of differentiating into osteoclasts.

Figure 1. Kaplan-Meier survival curves of ESFT

Figure 2. Luminex multiplex assay. Various inflammatory cytokines and chemokines were produced by TAMs.

To investigate the biological properties of TAMs in ESFT, we used mouse xenograft model, and isolated TAMs from xenografts. Immunostaining using F4/80 antibody revealed that a number of macrophages were infiltrating into mouse ESFT xenograft. TAMs were then isolated from xenografts by using CD11b-magnetic beads. Liver and spleen were used as a source of control monocytes (CoMs). FACS analysis revealed that about 90% of the cells were CD11b+CD45+ after the isolation, indicating that monocytes were successfully isolated from the tumor and normal tissue.

Luminex assay was performed for the screening of cytokine production. In the conditioned media of TAMs, higher levels of inflammatory cytokines and chemokines, including IL-6, KC, MCP-1 and RANTES, were observed. In contrast, almost no cytokine expression was detected in the conditioned medium of CoMs (Figure 2). This data indicates that TAMs are “activated” macrophages capable of producing various cytokines, which could affect tumor microenvironment.