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出版情報：American Journal of Pathology. 179 (3), pp.1157-1170, 2011-09. Elsevier / American Association of Pathologists
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Macrophage infiltration predicts a poor prognosis for the human Ewing sarcoma

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This work was supported by a Grant-in-Aid for Scientific Research (19390397) from the Japan

Society for the Promotion of Science (Y. Iwamoto), and a Grant-in-Aid for Clinical Research Evidence-Based Medicine and Cancer Research from the Ministry of Health, Labour and Welfare of Japan (Y. Iwamoto).

Number of text pages: 40

Number of tables: 5

Number of figures: 8

Number of supplemental figures: 3

A short running head: Macrophages predict a poor prognosis for EWS

INTRODUCTION

The Ewing sarcoma/primitive neuroectodermal tumor (EWS) is a small round-cell tumor type that typically arises in the bones of children and young adults. EWS is aggressive, with a tendency to metastasize to the lung and bone. As a result, these tumors are associated with the most unfavorable prognosis of all primary musculoskeletal tumors. The development of multimodal therapeutic regimens that include chemotherapy, irradiation, and surgery has increased the long-term survival rates for patients with localized disease. Smaller improvements, however, have been observed for patients with metastatic or recurrent disease.[1]

The symptoms of EWS at presentation include pain, swelling and fever, and laboratory findings such as elevated white blood cell counts, C-reactive protein (CRP) levels, and sedimentation rates are frequently observed. These findings indicate the existence of inflammation, and sometimes lead to a misdiagnosis of osteomyelitis and a delay in treatment.[2, 3] Biological mechanisms that account for the inflammation involved in EWS have remained uncertain. A better understanding of the characteristics of EWS may thus lead to the successful development of biologically targeted therapies in the future.

Recent studies have highlighted the importance of the cells from the tumor stroma. Blood vessels, fibroblasts, and such inflammatory cells as lymphocytes, neutrophils, and macrophages, are frequently observed in the tumor stroma. Interactions between stromal cells with tumor cells are thought to be essential for tumor malignancy.[4] For example, angiogenesis is clearly important for tumor growth and metastasis, and antibodies targeting vascular endothelial growth factor (VEGF) are

currently used to treat solid tumors.[5] In addition, the fibroblasts and neutrophils that infiltrate the tumor stroma have been demonstrated to be important for tumor initiation, growth, and metastasis.[6-8] Recently, tumor-infiltrating T-cells have been reported to be associated with a favorable prognosis in EWS.[9]

Among stromal cells, tumor-associated macrophages (TAMs) are known to play important roles in how a solid tumor will behave, including invasion, angiogenesis, and metastasis.[10] Macrophages have a wide phenotypic diversity, and can be classified into two activation phenotypes, M1 and M2.[11, 12] Classically activated M1 macrophages are inflammatory, and can exert cytotoxic activity. Alternatively, activated M2 macrophages are anti-inflammatory, and promote wound healing, angiogenesis, and tissue remodeling. TAMs often display features of M2 macrophages, and produce a number of cytokines and growth factors that promote tumor progression. TAMs also release a number of proteolytic enzymes that act to break down the extracellular matrix and basement membrane, allowing tumor cells to invade other tissues and endothelial cells to form vascular structures.[13] TAM accumulation is generally associated with a poor prognosis in patients with breast, prostate, bladder, and cervical cancers.[14-18] In patients with gastrointestinal stromal tumors, macrophages were more abundantly infiltrated in metastatic lesions compared with primary tumors.[19] In glioblastoma and melanoma, studies have shown that there is a significant correlation between the number of infiltrating macrophages and microvascular density (MVD) or tumor progression.[20, 21] On the other hand, in cases of osteosarcoma, activating macrophages with the muramyl tripeptide have been used as a cytotoxic therapy, and resulted in improvements in overall

survival, indicating that the TAMs in osteosarcoma have a suppressive effect on tumor progression.[22, 23]

Currently, little is known about the roles of TAMs in EWS. Lau and colleagues[24] reported that TAMs isolated from EWS arising in bones were capable of differentiating into osteoclasts, major mediators of tumor osteolysis. Additional studies are required to assess the functions of TAMs in EWS. In this study, we have isolated TAMs from mouse EWS xenografts, and investigated the characteristics of these cells. We also sought to determine the prognostic significance of TAMs in patients with EWS.

MATERIALS AND METHODS

Clinical samples

The study population consisted of 76 serial cases retrieved from the archives of the Department of Anatomic Pathology, Pathological Sciences, Graduate School of Medical Science, Kyushu University, Japan. The tissues were collected during primary tumor biopsy at diagnosis between 1978 and 2009. In each case, a diagnosis of EWS was made based on histological features. From these 76 cases, 27 cases were excluded because of a lack of availability of adequate tissue, and 8 cases lacked follow-up data, thus leaving 41 patients for the present study. All 41 cases presented with primary EWS, and 40 cases were treated with systemic multi-agent chemotherapy in combination with surgery/radiation. One patient refused the systemic chemotherapy after wide surgical resection, however, she has been disease free for 8 years after the surgery. Clinical data were

obtained by reviewing patient records, and survival data were collected during the summer of 2010. The Institutional Review Board at Kyushu University approved the use of human specimens for this study.

Immunohistochemistry

Antibodies specific for human CD68, CD31, and MIB1 were obtained from Dako (Glostrup, Denmark) and were used to evaluate human EWS clinical samples. To visualize macrophages and endothelial cells in mouse xenografts, anti-F4/80 (AbD Serotec, Oxfordshire, UK), anti-CD99 (Dako) and anti-CD31 (BMA Biomedicals, Basel, Switzerland) antibodies were used. Whole-section samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. After the sections were deparaffinized in xylene and rehydrated in a graded ethanol series, they were subjected to microwave pretreatment with citrate buffer (pH 6.0). After incubation with each antigen-specific antibody, samples were incubated with HRP-labeled goat anti-mouse antibodies (Dako). The reaction was visualized using the DAB substrate system (Wako, Osaka, Japan), and then samples were counterstained with diluted hematoxylin. To count the macrophages, an image with an area of 0.64 mm^2 was created from six different visual fields. The number of CD68 or F4/80 positive cells in six random field profiles was used for subsequent statistical analysis. To evaluate the MVD, CD31 positive vessels were counted in six random field profiles. Images were acquired using an AX70 microscope (Olympus, Tokyo, Japan) equipped with a DP72 camera (Olympus).

Cell lines

The RD-ES, SK-N-MC, and SK-ES-1 EWS cell lines were obtained from the American Type Culture Collection (Manassas, VA). WE-68 and VH-64 cells were kindly provided by Dr. Frans van Valen (Westfälische Wilhelms-University, Münster, Germany). These cells have been characterized previously.[25] TC-71 cells were obtained from the Coriell Institute (Camden, NJ). The murine macrophage RAW264.7 cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK). RD-ES, SK-ES-1, WE-68 and VH-64 cells were cultured in RPMI 1640 (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) at 37°C in an atmosphere of 5% CO_2. SK-N-MC, TC-71, and RAW264.7 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2.

Mouse xenografts

Female 6-week-old BALB/c nude mice were obtained from Charles River Japan (Fukuoka, Japan), and maintained in a specific pathogen-free environment throughout the experiment. Cells (5.0×10^6) derived from two EWS cell lines (RD-ES and TC-71) were resuspended in DMEM and Matrigel (BD Biosciences, Bedford, MA) at a 1:1 ratio, and injected into two subcutaneous locations on the back of each mouse. Tumor xenografts were excised four weeks after inoculation and then were used for further experiments. Experiments involving animals were performed in compliance with the guidelines established by the Animal Care and Use Committee of Kyushu University.

Isolation of CD11b$^+$ cells

CD11b$^+$ cells were isolated from mouse EWS xenograft tumors by magnetic sorting using CD11b MicroBeads (Miltenyi Biotec, Bergisch–Gladbach, Germany). Briefly, tissues were minced in 10 mL of DMEM, and collagenase L (Nitta Gelatin, Osaka, Japan) and DNase I (Roche, Basel, Switzerland) were added. The mixture was incubated for 30 min at 37°C under gentle agitation. Digestion was stopped with FBS, and the cell suspension was washed and passed through a 70 μm mesh nylon screen. The cells were incubated with CD11b MicroBeads for 15 min at 4°C and loaded onto a MIDIMACS column (Miltenyi Biotec) according to the manufacturer's instructions. Isolated CD11b$^+$ cells from xenografts were used as TAMs for further experiments. CD11b$^+$ cells were also isolated from mouse spleen and liver tissues, and used as control macrophages (CoMs). For cell surface staining, single-cell suspensions were incubated with FITC-conjugated anti-CD11b monoclonal antibodies (Miltenyi Biotec), APC-conjugated anti-CD11b, APC-conjugated anti-CD45 monoclonal antibodies, and PE-conjugated anti-F4/80 monoclonal antibodies (eBioscience, San Diego, CA) for 15 min at 4°C. The stained cells were run on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using the BD CellQuest software program (BD Biosciences).

Cytokine expression analysis

The expression of multiple cytokines was analyzed in CD11b$^+$ cells and EWS cell lines using Luminex 100 (Luminex, Austin, TX) according to the manufacturer's instructions. To collect

conditioned media, EWS cells (1×10^6/well) and $CD11b^+$ cells (5×10^5/well) were incubated in serum-free DMEM for 24 h and 72 h, respectively. The Human MultiAnalyte Profiling Base Kit A (R&D Systems, Minneapolis, MN) was used to examine EWS cells for the expression of interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-6, IL-8, IL-10, IL-17, basic fibroblast growth factor (bFGF), tumor necrosis factor (TNF)-α, interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1, regulated on activation normal T-cell expressed and secreted (RANTES), and VEGF. A multiplex mouse cytokine/chemokine kit (Millipore, Billerica, MA) was used to detect mouse IL-1α, IL-1β, IL-6, IL-10, IL-17, keratinocyte-derived chemokine (KC), MCP-1, MIP-1α, MIP-1β, RANTES, and VEGF.

To examine the effects of macrophages on VEGF production by EWS cells, 5×10^4 TAMs isolated from EWS xenografts were incubated in 500 μl of serum-free DMEM for 72 h. The serum-free DMEM or conditioned medium collected from TAMs was transferred to a monolayer of 1×10^5 RD-ES or TC-71 EWS cells, and collected after an additional 48 h incubation. The VEGF levels in the conditioned media were measured using a human VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems).

In vitro migration assay

A migration assay was performed using Transwell chambers (Costar, Cambridge, MA) with 6.5 mm diameter polycarbonate filters (8 μm pore size) as described previously.[25] In brief,

polyvinylpyrrolidone-free polycarbonate filters in the upper chamber were coated with type I collagen (Nitta Gelatin) and inserted into the lower chambers. RAW264.7 cells (2.0×10^5/well) were suspended in 200 µl of serum-free DMEM and seeded in the upper chamber. The lower chamber was filled with serum-free DMEM as a control sample or conditioned media obtained from $CD11b^+$ cells. In some experiments, EWS cells were plated in the lower chamber (2.0×10^5/well), and the VEGF receptor tyrosine kinase inhibitor IV (VEGFR-TKI) (Merck, Darmstadt, Germany) was added to both chambers at various concentrations (0, 0.1, 1, or 10 nM) to examine the involvement of VFGF signaling in cell migration. RAW264.7 cells were allowed to migrate for 4 h at 37°C, and the cells that migrated to the lower side of the filter were stained and counted as described previously.[24] Each experiment was repeated at least three times.

Osteoclastic differentiation assay

$CD11b^+$ cells were isolated and plated in 96 well plates at 5×10^4cells/well in 200 µl of DMEM (pH 7.4) containing 10% FBS, 50 ng/mL recombinant mouse macrophage colony stimulating factor (M-CSF; R&D Systems), and 50 ng/mL recombinant mouse receptor activator of NF-κB ligand (RANKL; R&D Systems). At the end of the culture period (4 days), cells were fixed, and their tartrate-resistant acid phosphatase (TRAP) activity was visualized using a TRAP staining kit (Primary Cell Co., Hokkaido, Japan). TRAP positive multinucleated giant cells containing three or more nuclei were counted under a microscope in four random field profiles. Each experiment was repeated at least three times.

RNA isolation and reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was extracted from each cell pellet using an RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand complementary DNA was generated from total RNA using a First Strand cDNA Synthesis Kit (Invitrogen) with random hexamer primers. Samples were then subjected to PCR amplification with oligonucleotide primers to detect the expression of RANKL and M-CSF mRNA (Table 1). The PCR products were electrophoresed through a 1.5% agarose gel (Invitrogen) containing ethidium bromide (Biotium, Hayward, CA). Real-time RT-PCRs were performed to quantitatively compare the expression level of each mRNA in $CD11b^+$ cells using the LightCycler system (Roche) with the SYBR Green I reagent (Takara, Tokyo, Japan). The expression levels of cathepsin K, triggering receptor expressed on myeloid cells 2 (TREM2), osteopontin, TRAP, nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and osteoactivin were examined using specific primers (Table 2). The mRNA expression levels were analyzed using the LightCycler version 3.5 software program (Roche). The data were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene.

***In vivo* macrophage depletion**

Liposome-encapsulated clodronate (Cl_2MDP-Lip) was prepared as described previously. [26-28] In brief, 11 mg of cholesterol (Sigma–Aldrich, St. Louis, MO) and 75 mg of phosphatidylcholine (Nacalai Tesque, Kyoto, Japan) were combined with 10 mL of 0.7 M Cl_2MDP (Sigma–Aldrich)

solution and sonicated gently. The resulting liposomes were washed three times to eliminate any free drug. Empty liposomes were prepared as control samples under the same conditions using phosphate-buffered saline (PBS) instead of Cl_2MDP. To assess the inhibitory effects of Cl_2MDP-Lip on RD-ES tumor proliferation, Cl_2MDP-Lip or PBS-Lip was administered 1 day before inoculation of the RD-ES cells. The mice received 200 μL of liposomes through a tail vein with a 28-gauge needle every 3 days.[28] Five mice were included in each group, and the lengths and widths of the tumors were measured every 3 days. Mice were sacrificed three weeks after the inoculation, and tumor masses were measured. All experiments were repeated three times.

Statistical analysis

Survival curves were calculated using the Kaplan–Meyer method, and log-rank tests were used for the survival analysis. Fisher's exact test was used to compare the categorized variables. The hazard ratios for risk factors for death were evaluated by a cox proportional-hazards regression analysis. *P* values < 0.05 were considered to be statistically significant. The data in graphs are presented as the means ± standard deviation (SD). Mann–Whitney U tests were used for two-group comparisons. All data analysis was carried out using a statistical software package (SAS, Cary, NC).

RESULTS

Identification and isolation of TAMs from EWS xenografts.

To determine whether macrophages infiltrate into EWS tumors, tumor xenografts were established by subcutaneously inoculating nude mice with RD-ES or TC-71 cells. Four weeks after inoculation, xenografts were excised and the infiltrating macrophages were examined. Xenografts were identified as EWS tumors by their characteristic CD99 staining (Figure 1A) and their specific mRNA expression of the EWS/FLI1 fusion gene (See Supplemental Figure S1 at http://ajp.amjpathol.org). 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 (Figure 1A). The flow cytometric analysis of collagenase-treated tumors revealed that approximately 2% of the xenograft cells were $CD11b^+$ and $F4/80^+$ (Figure 1B), thus suggesting that these cells were TAMs.

Using antibody-conjugated magnetic beads, we isolated TAMs from EWS xenografts based on their expression of CD11b. The $CD11b^+$ mononuclear cells were isolated as CoMs from the liver and spleen. A flow cytometric analysis demonstrated that approximately 90% of the isolated cells were positive for both CD11b and F4/80 (Figure 1C), thus suggesting that these cells could be used for further experiments.

Cytokine and chemokine expression by EWS-associated TAMs.

We examined the expression of various cytokines and chemokines by TAMs using the Luminex multiplex assay system, and compared the results with those observed in CoMs. As shown in Figure 2A, the expression levels of factors known to stimulate monocyte chemotaxis, including IL-6, MCP-1, KC, MIP-1β, and RANTES, were significantly upregulated in the conditioned media from

TAMs. In contrast, no marked cytokine expression was observed in the conditioned media from CoMs.

Because of the up-regulation of monokines in TAM cultures, we sought to examine whether TAMs induced the migration of monocytic cells. The transwell migration of monocytic RAW264.7 cells was increased in the presence of CoMs-conditioned media, and was further significantly enhanced in the presence of TAMs-conditioned media (Figure 2B). These data indicate that the TAMs in EWS are "activated" macrophages that secrete a number of cytokines/chemokines and induce the accumulation of monocytic cells.

We next examined the effect of TAMs on vascular endothelial cell tube formation, a critical process during angiogenesis. The formation of tube-like structures by microvascular endothelial cells increased in response to the RD-ES cell-conditioned medium, whereas no stimulatory effects were observed in the presence of TAMs-conditioned medium (data not shown).

Osteoclastic differentiation of TAMs in EWS.

The majority of EWS tumors arise in bone, and bone metastasis is often observed during the clinical course of these tumors. Because osteoclasts are critically involved in the development of bone tumors,[29] we examined the potential contribution of TAMs to osteoclastogenesis in EWS. To investigate their osteoclastic differentiation, TAMs were incubated for 4 days with soluble RANKL (sRANKL) and M-CSF, two factors crucial for osteoclastogenesis.[30] In the absence of sRANKL and M-CSF, no TRAP positive giant cells were developed from either TAMs or CoMs (See Supplemental

Figure S2A at http://ajp.amjpathol.org). However, in the presence of sRANKL and M-CSF, TRAP staining revealed the formation of multinucleated giant cells only from TAMs, thereby demonstrating that TAMs are capable of differentiating into osteoclasts (Figure 3A and See Supplemental Figure S2A at http://ajp.amjpathol.org). Significantly more TRAP positive giant multinucleated cells were developed from TAMs than from CoMs (Figure 3A).

To elucidate the mechanism involved in the enhanced osteoclastic differentiation of TAMs, we sought to examine the expression of RANKL and M-CSF in EWS cells. Some EWS cell lines have been shown to express RANKL,[24] however the expression of M-CSF has never been reported in EWS. Both RANKL and M-CSF mRNA expression were detected in all six of the examined EWS cell lines (Figure 3B). TAMs freshly isolated from EWS expressed such osteoclastic markers as cathepsin K, TREM2, osteopontin, TRAP, and osteoactivin (Figure 3C). An examination of the cell smear of TAMs revealed that freshly isolated cells were mononuclear, and no giant cells were observed (See Supplemental Figure S2B at http://ajp.amjpathol.org). Although very limited, TRAP activity was detected in some of TAMs (0.9% of the cells), while no TRAP positive cells were observed in CoMs (See Supplemental Figure S2B at http://ajp.amjpathol.org). The TRAP activity was also clearly detected in mononuclear cells that invaded EWS subcutaneous xenografts, even when these tumors had no contact with bone (Figure 3D). These observations suggest that some of the TAMs in EWS initiate osteoclastic differentiation within the tumor tissue.

VEGF recruits TAMs to EWS.

We next investigated the potential mechanisms underlying the recruitment of monocytes to EWS. The migration of RAW264.7 cells was significantly enhanced in co-cultures with RD-ES, TC-71 (Figure 4A), SK-N-MC and SK-ES-1 (data not shown). Therefore, we screened EWS cell lines for potential monocyte chemoattractants. A cytokine multiplex assay revealed that VEGF was secreted by all six of the EWS cell lines examined (Figure 4B). VEGF induces the migration of monocytic cells that express the VEGF receptor Flt-1.[31] As previously shown by Matsumoto et al,[31] RAW264.7 cell migration was dose-dependently stimulated by VEGF (data not shown). Moreover, blocking VEGF-receptor signaling reduced EWS-induced RAW264.7 cell migration by 65% (Figure 4C).

Because VEGF production is induced in various tumor cells by inflammatory stimuli,[21] we cultured EWS cells with TAMs-conditioned medium, and examined VEGF production using an ELISA. Increased VEGF secretion was observed when the RD-ES or TC-71 cells were stimulated with TAMs-conditioned medium (Figure 4D). These results demonstrated that the recruitment of TAMs to EWS is, at least in part, dependent on EWS-derived VEGF, the secretion of which is upregulated in the presence of TAMs.

Effects of macrophage depletion on the development of EWS xenografts.

To investigate the involvement of TAMs in the development of EWS, we used Cl_2MDP-Lip[27, 28] to decrease the number of monocytes/macrophages in mouse EWS xenografts. Compared with PBS-Lip, Cl_2MDP-Lip significantly inhibited the development of xenografts (Figure 5A), whereas no inhibitory effects on the proliferation of RD-ES cells were observed *in vitro* (data not shown).

Twenty-one days after inoculation, xenografts were excised and examined. Although the changes were not significant, the average xenograft weight tended to be lower in mice treated with Cl_2MDP-Lip (Figure 5B). An immunohistochemical analysis of mice treated with PBS-Lip revealed numerous F4/80 positive macrophages in the tumors, whereas fewer infiltrating macrophages were observed in tumors treated with Cl_2MDP-Lip (Figure 5, C and D). Additionally, the tumor vasculature was significantly decreased in mice treated with Cl_2MDP-Lip (Figure 5, C and D), thus suggesting that the inhibition of angiogenesis contributed to the reduced tumor growth.

The association between infiltrating macrophages and a poor clinical outcome in EWS.

Finally, we investigated whether infiltrating macrophages were associated with the clinical outcomes of patients with EWS. Anti-CD68 antibodies were used to quantify the number of TAMs in EWS clinical samples. Representative images of EWS samples with CD68 positive, tumor-infiltrating macrophages are shown in Figure 6, A and B. The signals were localized in the membrane and cytoplasm, but not in the nucleus, of these cells. To further confirm the identity of CD68 positive cells in EWS as macrophages, we performed double fluorescent immunostaining for CD68 and CD14 (See Supplemental Figure S3 at http://ajp.amjpathol.org). An examination of 10 different EWS clinical samples revealed that 97% of the CD68 positive cells were also positive for CD14, indicating that the CD68 positive cells in EWS are macrophages.

A Kaplan–Meier survival analysis was performed to determine the prognostic significance of TAMs and other clinical parameters in 41 cases of EWS. The clinical characteristics of the cases are

provided in Table 3. A higher extent of macrophage infiltration (CD68 numbers > 30/high power field [HPF]) and a higher MVD (> 10/HPF; Figure 6, A and B) were associated with a poorer overall survival (Figure 7, A and B). In addition, elevated CRP (> 0.2 mg/dL) and white blood cell (WBC) counts (> 6800 cells/μL) were also associated with a poorer prognosis (Figure 7, C and D). A higher macrophage infiltration rate was also significantly associated with a higher MVD (odds ratio [OR], 8; 95% confidence interval [CI], 1.9 to 33.2; $p = 0.0044$), elevated serum CRP (OR, 16; 95% CI, 3.2 to 78.3; $p = 0.0003$), and WBC counts (OR, 8; 95% CI, 1.9 to 30.0; $p = 0.0048$) (Table 4). Neither the serum levels of CRP nor the WBC counts correlated with the tumor size (data not shown). As reported previously, more frequent MIB1 expression (MIB1 index ≥ 40; Figure 6B) and larger tumor size (≥ 8 cm) were significantly associated with a poorer prognosis (Figure 7, E and F).[1, 32, 33] In addition, increased serum lactate dehydrogenase (LDH) levels (> 340 IU/L) tended to be associated with a poor prognosis, although statistical significance was not observed (data not shown). Age (≤ 18 years), sex, and the position of the tumor was not found to be associated with overall survival (data not shown). We also performed a univariate and multivariate analysis with variables including the CD68 numbers, tumor size, and treatment with multi-agent chemotherapy. In a multivariate analysis, the CRP levels, WBC counts, and MDV were excluded from the variables because of their strong association with the CD68 numbers (Table 5). Both the CD68 numbers and tumor size were identified to be significant factors by the univariate analysis, however, only the CD68 numbers remained as a significant predictor of a poor prognosis in the multivariate analysis (Table 5).

DISCUSSION

Through the production of growth factors, cytokines/chemokines, and proteases, TAMs play important roles in tumor invasion, angiogenesis, and metastasis.[34, 35] Infiltrating TAMs were found to be associated with systemic inflammation, enhanced tumor vasculature, and poor clinical outcomes in patients with EWS, thus suggesting that TAMs could be used as a prognostic factor for this family of tumors (Figure 8). The prognostic importance of high infiltration of TAMs in EWS was also confirmed by the multivariate analysis (Table 5). Consistent with previous reports,[24, 36] TAMs isolated from EWS xenografts had a number of distinctive characteristics with regard to cytokine production and osteoclastogenesis, when compared with control macrophages. This is the first report of the association between a poor prognosis and the biological properties of TAMs in EWS.

Tumor-host immune interactions within the tumor microenvironment may modulate tumor progression, and both tumor-protective and tumor-promoting features of the immune response have been described.[37] With regard to the protective effects, tumor-infiltrating T-cells are reported to be associated with a favorable prognosis in EWS,[9] and various studies have been undertaken to develop immunotherapeutic strategies for advanced stage EWS.[38-40] On the other hand, the association of inflammation with tumor progression is also well-documented in several tumor types,[41] and TAMs are thought to be major regulators of inflammation in various tumors.[35] Elevated serologic inflammatory markers, such as CRP levels and WBC counts, are known to be characteristic of EWS.[1, 32, 42] Although both the elevated CRP level and WBC count were significantly associated with the higher infiltration of TAMs (Table 4), the squared correlation coefficients (R^2) were relatively

small, at 0.17 and 0.28, respectively (data not shown), thereby indicating no obvious correlations between TAMs and the serum serological inflammatory markers. This suggests that other factors, such as lymphocytes, may be involved in the development of inflammation in EWS.[9] Further studies are required to better understand the roles of inflammatory cells during the progression of EWS.

The factors in serum are useful as diagnostic and/or prognostic markers. Bacci and colleagues examined 579 cases of EWS and reported an association between the serum LDH levels and prognosis.[43] Although we observed that a higher LDH level may predict a poorer prognosis, no statistically significant association was observed, probably because of the relatively smaller number of cases evaluated in our study (N = 41). Instead, elevated serum CRP levels and WBC counts were significantly associated with a poorer prognosis in the present study, providing potential new prognostic markers that can be easily obtained in the clinical setting.

Tumor angiogenesis is often a limiting factor for tumor growth and metastasis, and correlates with a poor prognosis in carcinomas of the breast, bladder, and cervix.[16, 18, 44] For EWS, Mikulic et al[45] examined 27 EWS cases and reported that a lower MVD was associated with a tendency toward better outcomes, although their results were not statistically significant. In addition to regular blood vessels, EWS tumor cells have also been reported to contribute to an increased tumor blood supply and be associated with a poor prognosis, by forming a vascular-like tube formation via endoglin signaling. [46,47] In this study, we observed a significantly association between infiltrating macrophages and MVD in 41 EWS cases (Figure 6, A and B, Table 4). Decreasing the macrophage numbers using Cl_2MDP reduced the tumor vascularity and slowed tumor growth in mouse EWS xenografts (Figure

5, A-D). Although TAMs in EWS did not directly stimulate endothelial tube formation (data not shown), they significantly stimulated EWS to produce VEGF (Figure 4D), one of the most potent inducers of angiogenesis. Our observation suggests that TAMs in EWS indirectly promote angiogenesis by stimulating VEGF production from EWS tumor cells, thus resulting in tumor progression (Figure 8).

VEGF plays an indispensable role in the growth of EWS. Small interfering RNA targeting VEGF has been used to inhibit EWS growth in a xenograft mouse model.[48] Patients with EWS have been demonstrated to have increased serum VEGF levels compared with healthy control subjects,[49] and a histologic evaluation revealed that elevated VEGF expression in EWS correlates with poorer clinical outcomes.[50] Although both macrophages and tumor cells can be a source of VEGF,[51] we observed no detectable VEGF production from TAMs in EWS (Figure 2A). This may explain why TAMs-conditioned media did not stimulate the proliferation or tube formation by endothelial cells, two activities that primarily depend on VEGF.[52] Activated macrophages produce inflammatory cytokines such as IL-1 and MCP-1, which can enhance VEGF production by tumor cells.[21, 26, 53, 54] These reports, in concert with our present observation, suggest that the angiogenesis in EWS is predominantly regulated by EWS-derived VEGF, the expression of which is significantly upregulated in the presence of TAMs (Figure 4D).

The detailed mechanisms underlying macrophage accumulation in EWS are not clear. VEGF may play a role in the initial recruitment of TAMs, because EWS-induced migration of monocytic RAW264.7 cells was significantly reduced in the presence of a VEGF receptor inhibitor (Figure 4C).

VEGF stimulates monocyte migration via the Flt-1 receptor signaling pathway,[31] and contributes to the accumulation of TAMs in breast cancer.[55] A recent report revealed that VEGF expression was upregulated by a EWS/FLI1 fusion gene,[56] which may explain why VEGF was secreted by all six of the EWS cell lines examined in the present study (Figure 4B).

In addition to VEGF, cytokines/chemokines play important roles in the accumulation of TAMs. The expression levels of MCP-1, MIP-1, and RANTES correlate with the number of TAMs in various cancers.[57] These monocyte/macrophage chemoattractants are produced not only by tumor cells, but also by stromal cells, including macrophages themselves.[6] TAMs in EWS expressed several soluble inflammatory factors, including IL-6, GRO1 (mouse KC), MCP-1, MIP-1β, and RANTES (Figure 2A), all of which are capable of stimulating monocyte chemotaxis.[58-62] These factors may contribute to the recruitment and accumulation of macrophages in EWS in an autocrine/paracrine manner. In addition to recruiting macrophages, cytokines/chemokines regulate the development of the tumor microenvironment. For example, GRO1 has been implicated in regulating stromal fibroblasts during ovarian tumorigenesis,[63] and promoting breast cancer metastasis.[64] RANTES, which is secreted from mesenchymal stem cells in tumors, reportedly promotes breast cancer metastasis.[6] MCP-1 regulates angiogenesis in gastric cancer via macrophage recruitment.[65] The roles of these factors during the development of EWS require further elucidation.

During bone tumor development, bone matrix is absorbed and degraded primarily by osteoclasts,[29] which are specialized cells that differentiate from peripheral circulation- or bone marrow-derived monocytic cells. The serum TRACP 5b levels and the presence of active osteoclasts

are positively associated with the aggressiveness of primary osteosarcoma.[66] Lau et al[24] reported that TAMs in EWS arising in bones are capable of differentiating into osteoclasts via both RANKL-dependent and RANKL-independent pathways. Compared with control macrophages, TAMs exhibited enhanced osteoclastogenesis (Figure 3A), thus suggesting that TAMs may promote both tumor progression and osteolysis in EWS.

Additionally, we detected the expression of the osteoclastic markers cathepsin K, TREM2, osteoactivin and osteopontin in TAMs (Figure 3C), thus suggesting that osteoclastic differentiation had been initiated in this cell population, possibly as a result of the expression of RANKL and M-CSF by the EWS cells (Figure 3B). VEGF may enhance osteoclastogenesis by up-regulating not only RANKL in EWS, but also RANK in osteoclast precursor cells.[67] Furthermore, various factors secreted by TAMs, including IL-6, MCP-1, MIP-1β and RANTES, may also enhance osteoclastic differentiation in an autocrine manner.[59, 60, 68-70] Intriguingly, cathepsin K and osteoactivin are reported to promote breast tumor progression.[71, 72] TAMs are reported to express osteoactivin, which could be speculatively linked to a tumor tissue remodeling function and matrix metalloproteinase activation.[35] Osteopontin is reportedly involved in the progression of various tumors, such as prostate cancer, lung cancer, breast cancer, pancreatic cancer, and hepatocellular carcinoma.[73, 74] An increased expression of cathepsin K, osteoactivin and osteopontin in TAMs may also play a role in the progression of EWS.

In conclusion, this study revealed a significant association between macrophage infiltration in EWS and clinical outcomes. TAMs appear to enhance the progression of EWS by stimulating both

angiogenesis and osteoclastogenesis, processes mediated by various cytokines and chemokines (Figure 8). TAMs and the various factors that they produce may provide new therapeutic targets for EWS.

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Figure legends

Figure 1. Identification and isolation of TAMs from mouse EWS xenograft tumors

A: Immunohistochemical staining for CD99 and F4/80 in mouse EWS xenografts. Nude mice were subcutaneously inoculated with RD-ES or TC-71 EWS cells. EWS tumors were characterized by using hematoxylin-eosin (H/E) staining and CD99 immunostaining. Representative images of EWS xenografts infiltrated by F4/80 positive macrophages are shown (arrow). **B:** The surface marker expression on dissociated xenograft cells. After mincing, xenograft cells were dissociated using collagenase and DNase, and subjected to a flow cytometric analysis. **C:** The surface marker expression on isolated $CD11b^+$ cells. After isolating cells using anti-CD11b beads, the cells from EWS tumor xenografts (TAMs) or liver (CoMs) were subjected to a flow cytometric analysis. Scale bar: 20 μm (**A**).

Figure 2. Cytokine expression by TAMs in mouse EWS xenograft tumors

A: The chemokine expression by isolated $CD11b^+$ cells. TAMs or CoMs were incubated in serum-free DMEM for 72 h, and the conditioned media were examined using a Luminex multiplex assay system. The results show the means ± SD (*: $P < 0.05$). **B:** The effect of the conditioned media on monocytic migration was examined using a Transwell system. Monocytic RAW264.7 cells were added to the upper well, and TAMs- or CoMs-conditioned medium was placed in the lower well.

After 4 h of incubation, the cells that had migrated to the bottom surface were stained and counted. The results show the means ± SD (*: $P < 0.05$; **: $P < 0.01$).

Figure 3. Osteoclastic differentiation of TAMs in EWS

A: The induction of osteoclastic differentiation. TAMs or CoMs were incubated with sRANKL and M-CSF for 4 days. Osteoclastic differentiation was visualized by TRAP staining (left). The TRAP positive multinucleated giant cells were counted (right). The results show the means ± SD (*: $P < 0.05$). **B:** RT-PCRs were performed to detect the expression of RANKL and M-CSF mRNA in six EWS cell lines. **C:** Quantitative RT-PCRs were performed to detect osteoclastic differentiation in $CD11b^+$ cells. All expression levels were normalized based on the expression of GAPDH. The data show the relative expression in TAMs (gray bars) compared with CoMs (white bars). The results show the means ± SD (*: $P < 0.05$). **D:** TRAP staining of EWS xenografts that had developed from RD-ES or TC-71 cells. Sections were counterstained with diluted methyl green solution.

Scale bars: 20 μm (**A**,), 50 μm (**D**).

Figure 4. EWS cell lines stimulate monocyte migration via VEGF signaling

A: The migration of monocytic cells was examined using a Transwell system. The lower wells were filled with serum-free medium, RD-ES cells, or TC-71 cells, and the RAW264.7 cell migration to the bottom surface of the Transwell was assessed. The results show the means ± SD (**: $P < 0.01$). **B:** The Luminex multiplex assay system was used to screen for chemotactic factors that were produced

by EWS cells. **C:** The inhibitory effects of VEGFR-TKI on the migration of RAW264.7 cells. RAW264.7 cells were co-cultured with RD-ES cells, and their migration to the bottom surface of the Transwell in the presence of VEGFR-TKI was assessed. The results show the means ± SD (**: $P < 0.01$). **D:** Quantification of VEGF secretion by EWS cells. RD-ES or TC-71 cells were stimulated with TAMs-conditioned media (CM) for 48 h, and the VEGF levels in conditioned media from EWS cells were examined using a human VEGF ELISA kit. Serum free DMEM was used as negative control. The results show the means ± SD (*: $P < 0.05$).

Figure 5. The effects of macrophage depletion in an EWS xenograft model

A: (Left) Cl_2MDP-Lip (white squares) or PBS-Lip (black circles) was administered intravenously to nude mice 1 day before they were inoculated with RD-ES cells. Mice received 200 μL of liposomes through the tail vein every 3 days. The length and width of the tumors were measured for 3 weeks after inoculation. (Right) The dot plot for the tumor volumes at 20 days after inoculation is indicated. The tumor volumes of the group treated with Cl_2MDP-Lip were significantly lower than those of the PBS-Lip group. Five mice were used for each group. The results show the means (dot-lines) ± SD (straight lines) (*: $P < 0.05$). **B:** The tumors were excised and weighed 3 weeks after inoculation. **C:** Immunohistochemical staining of macrophages and the tumor vasculature in EWS xenografts. Infiltrating macrophages were visualized (arrow) using anti-F4/80 antibodies (left). The tumor vasculature was visualized (asterisk) using anti-CD31 antibodies (right). **D:** The mean numbers of F4/80 positive macrophages and CD31 positive vessels in six random field profiles were used for the

subsequent statistical analyses (Mann–Whitney U test). The results show the means ± SD (**: $P < 0.01$). Scale bars: 20 μm (**C**)

Figure 6. Immunohistochemical staining of human EWS sections

Representative staining of macrophages, the tumor vasculature and MIB1 in EWS samples. Paraffin sections were immunohistochemically stained with anti-CD68, anti-CD31, and anti-MIB1-antibodies, and visualized using the DAB substrate system. Counterstaining was then performed with diluted hematoxylin. Prominent tumor microvasculature and MIB1 expression were evident in the cases with higher macrophage infiltration (CD68 numbers > 30/HPF; cases 36 [DOD] and 37 [DOD]) (**B**), compared with the cases with lower macrophage infiltration (CD68 numbers ≤ 30/HPF; cases 4 [CDF] and 6 [NED]) (**A**). HPF, high power field. Scale bars: 20 μm (**A** and **B**)

Figure 7. The association between macrophage infiltration and a poor prognosis in EWS

A-F: Kaplan–Meier survival curves are shown for all patients based on CD68 positive macrophage infiltration (low CD68 numbers: ≤ 30/HPF; high CD68 numbers: > 30/HPF) (**A**) ,MVD (low: ≤ 10/HPF; high: > 10/HPF) (**B**), serum CRP levels (low: ≤ 0.2 mg/dL; high: > 0.2 mg/dL) (**C**), WBC counts (low: ≤ 6800 cells/μL; high: > 6800 cells/μL) (**D**), MIB1 expression (low MIB1 index values: < 40; high MIB1 index values: ≥ 40) (**E**), and tumor size (small: < 8cm; large: ≥ 8) (**F**). Log-rank tests were performed to determine the statistical significance, with *P* values < 0.05 defined as significant (*: $P < 0.05$, **: $P < 0.01$).

Figure 8. A model for the TAM-mediated modulation of the EWS microenvironment

TAMs play important roles as modulators of inflammation, angiogenesis and osteoclastogenesis during EWS development. 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 in EWS. TAMs stimulate tumor angiogenesis by enhancing VEGF production from tumor cells, resulting in a poorer prognosis. The enhanced osteoclastogenesis induced by TAMs enhances bone tumor progression, and may affect the prognosis of patients with EWS.

Table 1. Human and mouse primer sequences used for conventional RT-PCR

Gene		Primer sequence	Amplicon Size (bp)
GAPDH (H)	forward	5’-ACCACAGTCCATGCCATCAC-3’	452
	reverse	5’-TCCACCACCCTGTTGCTGTA-3’	
GAPDH (M)	forward	5’-GTGGCAAAGTGGAGATGGTTGCC-3’	290
	reverse	5’-GATGATGACCCGTTTGGCTCC-3’	
M-CSF (H)	forward	5’-CAGTTGTCAAGGACAGCAC-3’	671
	reverse	5’-GCTGGAGGATCCCTCGGACTG-3’	
RANKL (H)	forward	5’-GCCAGTGGGAGATGTTAG-3’	487
	reverse	5’-TTAGCTGCAAGTTTTCCC-3’	
EWS/FLI1 (H)	forward	5’-CCACTAGTTACCCACCCCAAACTG-3’	332 (type1)
	reverse	5’-GTGATACAGCTGGCGTTGGCG-3’	398 (type2)

H, human; M, mouse

Table 2. Mouse primer sequences used for real time RT-PCR

Gene		Primer sequence	Amplicon Size (bp)
GAPDH	forward	5’-GGAAGGCCATGCCAGTGAGC-3’	194
	reverse	5’-CATTGTGGAAGGGCTCATGA-3’	
Cathepsin K	forward	5’-TGTATAACGCCACGGCAAA-3’	195
	reverse	5’-GGTTCACATTATCACGGTCACA-3’	
TREM2	forward	5’-CTGCACTTCAAGGGAAAAGC-3’	203
	reverse	5’-CAGTGCTTCAAGGCGTCATA-3’	
Ostepontin	forward	5’-GGCATTGCCTCCTCCCTC-3’	69
	reverse	5’-GCAGGCTGTAAAGCTTCTCC-3’	
TRAP	forward	5’-TACCTGTGTGGACATGACC-3’	151
	reverse	5’-CAGATCCATAGTGAAACCGC-3’	
NFATc1	forward	5’-AATAACATGCGAGCCATCATC-3’	109
	reverse	5’-TCACCCTGGTGTTCTTCCTC-3’	
Osteoactivin	forward	5’-TCCCTGGCAAAGACCCAGA-3’	107
	reverse	5’-TTTGTACAGCAAGATGGTAACCATG-3’	

Table 3. The relationship between the tumor CD68 expression and the clinicopathological characteristics of the EWS

Variable	**CD68 numbers**	
	low (≤30)	**high (>30)**
Follow-up period (median)	15-138 (60.4) months	7-181 (41.3) months
Sex		
Male	9	9
Female	12	11
Age, y		
≤18	12	9
>18	9	11
Range (median)	5-68 (23)	8-74 (23)
Location		
Extremities	9	8
Trunk	12	12
Origin site		
Skeletal	10	11
Extraskeletal	11	9
Metastasis at diagnosis		
Bone	1	2

Lung	3	2
Bone + lung	0	1
Other	0	1
Tumor size		
<8cm	12	7
≥8cm	9	13
CD31 vessel number		
≤10	14	4
>10	7	16
Status		
CDF	10	4
NED	1	0
AWD	4	0
DOD	6	16
Systemic multi-agent chemotherapy		
Yes	20	20
No	1 (Status: CDF)	0
Surgery and/or Radiation	21	20
Initial laboratory parameters		

CRP (mg/dL) (median)	0-3.5 (0.5)	0.1-27 (6.4)
WBC (/μL) (median)	4010-12100 (6507)	3120-12570 (7855)
LDH (IU/L) (median)	150-7100 (613)	235-4973 (742)

CDF, continuous disease free; NED, no evidence of disease; AWD, alive with disease; DOD, dead of disease

Table 4. The results of the univariate and multivariate analyses for overall survival

	Univariate analysis		Multivariate analysis	
Variable	**Hazard ratio**	**p value**	**Hazard ratio**	**p value**
CD68 numbers				
low (≦30)	0.2772	0.0044**	0.3400	0.0235*
high (>30)	1		1	
Tumor size				
<8cm	0.4045	0.0403*	0.5804	0.2447
≥8cm	1		1	
Multi-agent chemotherapy***				
VDC-IE (n=11)	0.9521	0.9301	1.1129	0.8528
Other (n=29)	1		1	

***except surgery alone

VDC-IE, vincristine-doxorubicin-cyclophosphamide-ifosfamide-etoposide

*: $p<0.05$, **: $p<0.01$