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Expression and Localization of Extracellular Matrix Metalloproteinase Inducer in Giant Cell Tumor of Bone

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Abstract Matrix metalloproteinases (MMPs) are regarded as a significant regulator in tumor invasion and metastasis. Previous studies have shown that extracellular matrix metalloproteinase inducer (EMMPRIN) in tumor cells induces the synthesis of MMPs. EMMPRIN is abundantly present on the surface of tumor cells and stimulate adjacent stromal cells to synthesize MMPs to induce tumor progression. Giant cell tumor (GCT) of bone is a benign but locally aggressive primary neoplasm of bone. The spindle-shaped mononuclear stromal cells are considered to be the tumor components of GCT, which are capable of inducing osteoclast formation by recruiting the circulating monocyte and macrophage. In this study, we proposed that EMMPRIN is associated with the biological progression and aggressiveness of GCT. We have conducted semi-quantitative RT-PCR to determine the correlation of EMMPRIN expression with the clinical stage of GCT. We have also examined the cellular localization of EMMPRIN in GCT using in-situ hybridization (ISH) and Immunohistochemistry (IH). The results showed that EMMPRIN was present in GCT and its mRNA levels were associated with the clinical stage of GCT. Higher expression level of EMMPRIN was observed in GCT with advanced stage (stage III). There was a great significance ($P < 0.05$) of EMMPRIN expression between stage I & II and stage III GCTs. Both ISH and IH demonstrated that EMMPRIN is present at the multinuclear osteoclast-like giant cells of GCT, with strong immunostaining on the cell membrane. The stromal-like tumor cells were also positively stained but the intensity was weaker. Interestingly, the production of EMMPRIN in osteoclast-like cells of GCT seems to be regulated by stromal-like tumor cells. Receptor activator of NF-κB ligand (RANKL), which has been previously shown to be produced by the stromal-like tumor cells for the recruitment of osteoclast-like giant cells in GCT, enhanced the expression of EMMPRIN mRNA during the differentiation of macrophage-like $RAW_{264.7}$ cells into osteoclasts. In short, our studies suggest that EMMPRIN may be an important regulatory factor involved in the biological behaviors of GCT. J. Cell. Biochem. 89: 1154–1163, 2003.

Key words: giant cell tumor (GCT) of bone; EMMPRIN; MMPs; TIMPs; RANKL

Giant cell tumor (GCT) of bone is an osteoclast-like giant cell rich benign primary neoplasm that is usually solitary but aggressive. It is considered to be the only benign tumor, which has the ability of metastasis. GCT occurs in young adults aged 20–50 years, and females are slightly more often affected than males. Patients almost always present with pain, and a few with a pathologic fracture [McCarthy and Frassica, 1998]. The common location of the neoplasm in over 75% of cases is the epiphysial site of a long tubular bone, e.g., distal femur, proximal tibia, and distal radius, [Schajowicz, 1994], and the pulmonary metastasis occur rarely in about 1–2% of patients. We, and others have previously demonstrated the stromal-like cells, which have the ability to recruit macrophage and multinucleated osteoclast-like giant cells, are putative tumor cells of GCT [Goldring et al., 1987; Doussis and Puddle, 1992; Zheng and Fan, 1994; Zheng et al., 1995;

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Zheng et al., 1998; Zheng et al., 1999; Atkins et al., 2000; Huang et al., 2000]. Various histological gradings of GCT developed for the prediction of recurrent behaviour and metastatic potential have been shown to lack value, [Dorfman and Czeriniak, 1998] whereas Enneking's Clinical Classification of GCT seems to reflect the aggressiveness of GCT [Enneking, 1983]. The Clinical Classification of GCT reflects the degree of locally aggressiveness, including pathological fractures, local recurrence, subchondral-plate damage and soft tissue penetration [Enneking, 1983].

Extracellular matrix metalloproteinase inducer (EMMPRIN), also named as CD147, Basigin, Tumor Collagenase Stimulatory Factor (TCSF) or M6 antigen, was initially characterized as a transmembrane glycoprotein that is enriched on the cell surface of malignant human tumors [Ellis et al., 1989; Nabeshima et al., 1991; Guo et al., 1997]. The molecular structure of this glycoprotein has two extracellular immunoglobulin-like domains including a putative transmembrane domain and a 39-amino acid cytoplasmic domain [Biswas et al., 1995; Sun and Hemler, 2001]. It has been previously demonstrated that EMMPRIN produced by tumor cells has the ability to stimulate adjacent stromal cells to synthesize several matrix metalloproteinases (MMPs), a family of the prominent enzymes that degrade different components of the extracellular matrix and regulate tissue remodeling, tumor invasion and metastasis [Liotta et al., 1991; Hart and Saini, 1992; MacDougall and Matrisian, 1995; Sameshima and Nabeshima, 2000]. A number of studies have shown that the production and activation of most MMPs in fibroblasts are primarily stimulated by both native and recombinant purified EMMPRIN in a variety of human malignant tumors including those of brain, breast, lung, oral, and skin cancers [van den Oord and Paemen, 1997; Lim et al., 1998; Caudroy et al., 1999; Bordador et al., 2000; Dalberg and Eriksson, 2000; Sameshima and Nabeshima, 2000; Sun and Hemler, 2001; Kanekura and Chen, 2002; Taylor and Woodfield, 2002]. Based on those evidences, EMMPRIN may play a regulatory role in promoting MMP-dependent tumor aggression.

MMPs are regarded to play a significant regulatory role in tumor aggressiveness of GCT [Sasaguri et al., 1992; Rao et al., 1997]. There are greater than 20 human MMPs being identified and categorized into collagenases, gelatinases, stromelysins, membrane-type, and others based on their domain structure [Kleiner and Stetler-Stevenson, 1999]. Recent studies have shown the presence of several MMPs in GCT, including interstitial collagenase (MMP-1), gelatinase A (72 kDa gelatinase, MMP-2) and gelatinase B (92kDa geltinase, MMP-9) [Rao et al., 1995; Rao et al., 1997; Masui and Ushigome, 1998; Teti et al., 1998]. Inhibition of MMP production may have a potential therapeutic approach in tumor progression. Tissue inhibitors of metalloproteinase (TIMPs) are believed to play an important role in extracellular inhibition of the production of MMPs [Joronen et al., 2000]. However, to date, the accurate correlation between the EMMPRIN-MMP interaction and the neoplastic aggression of GCT remains unknown.

In the present study, we investigated the expression and localization of EMMPRIN in the GCT tissues and examined whether EMMPRIN, MMP-1, 2, and TIMP-1, 2 are associated with the clinical stage of GCT. We also analyzed the regulation of EMMPRIN in macrophage-like cell culture by RANKL. The findings suggested that EMMPRIN mRNA was synthesized in GCT and was up-regulated by RANKL during osteoclastogenesis. Moreover, its expression levels are significantly correlated with aggressive potential of the tumor progression. EMMPRIN may be a regulatory factor involved in processing MMP-dependent tumor behaviors of GCT.

MATERIALS AND METHODS

Materials

All human GCT tissues from 19 individual cases were freshly obtained from surgical removal of tumors at the Sir Charles Gairdner Hospital, Perth, WA, Australia, or the Prince of Wales Hospital, Hong Kong, China. Their clinical details at presentation are summarized in Table I. Among these specimens, a series of semi-quantitative revere transcription-polymerase chain reaction (RT-PCR) studies were performed with 19 cases to analyze of EMMPRIN, MMP-1, 2, and TIMP-1, 2 in GCT. The imprint slides from eight cases were used for in-situ hybridization (ISH) and immunohistochemistry (IH). GCT stromal cell cultures were obtained from two cases of GCT. Human osteosarcoma cell line U2OS and mouse

TABLE I. Clinical Details of GCT Cases

Cases	Sex	Localization	Clinical stage
1	Female	Proximal tibia	III
2[a]	Male	Femur	III
3	Male	Femur	III
4[a]	Female	Unknown	I
5	Female	Humerus	III
6[a]	Male	Distal femur	II
7	Male	Unknown	I
8	Female	Unknown	I
9[a]	Male	Unknown	I
10[a]	Female	Proximal pelvis	II
11[a]	Male	Proximal tibia	III
12	Male	Proximal tibia	II
13[a]	Female	Femur	II
14	Male	Femur	II
15[a]	Female	Radius	I/II
16[a]	Female	Humerus	I/II
17	Female	Tibia	I/II
18[a]	Female	Femur	I/II
19[a]	Male	Femur	II

[a]These cases were also examined in the PCR analysis of MMP and TIMP in GCT.

macrophage cell line $RAW_{264.7}$ were obtained from American Type Culture Collection (Rockville, MD) and normal human fibroblasts were used as a control. Dulbecco's minimum essential medium (DMEM) and fetal bovine serum (FBS) were purchased form TRACE (Sydney, Australia). Monoclonal antibody (mAb) 8G6 against human EMMPRIN was obtained from Dr. Hemler, ME, the DANA-FARBER Cancer Institute, Harvard Medical School, Boston, USA [Berditchevski et al., 1997]. The mAb to human MCP-1 or KP-1 (CD68), a monocyte-macrophage marker, was obtained from DAKO (Botany, Australia) and used as positive controls of osteoclast-like giant cells [Zheng et al., 1995]. All other chemical reagents used were of the analytical grade available.

RT-PCR

Total RNA was isolated from human GCT tissues by using RNAzol B (RNA-Bee™) according to the manufacture's instruction. A total of 17 cases of GCT were examined for the presence of EMMPRIN mRNA. Two micrograms of mRNA from each sample were used to synthesize the first-stand complementary DNA (cDNA), using with Olido-dt_{18} primer (Genset Oligos) and M-MLV reverse transcriptase.

PCR amplification was performed using 1–2 U of Taq polymerase (Promega, USA) with 0.4 mM of target-specific primers (Table II), 5 μM of dNTP in 10 × PCR buffer with 2 μM of

TABLE II. Sequences of Selective PCR Primers

Region	Sense primer	Antisense primer	Product size (bp)	Annealing temperature (°C)	Cycle preformed
h EMMPRIN[a]	5′ AAGCTTAATAGGAATCATGGCGGCTG 3′	5′ TCTAGACAGGAAGAGTTCCTCTGGC 3′	819	55	30
MMP-1	5′ TGCTCATGCTTTTCAACCAG 3′	5′ GAACATCACTTCTCCCCGAA 3′	361	58	30
MMP-2	5′ CACTTTCCTGGGCAACAAAT 3′	5′ TGATGTCATCCTGGGACAGA 3′	273	58	30
TIMP-1	5′ CGGGATCCAGAGAGACACCAGAGAA 3′	5′ GCTCTAGACAGGCTATCTGGGACCGCA 3′	660	58	30
TIMP-2	5′ GATGCACATCACCCTCTGTG 3′	5′ GTCGAGAAAACTCCTGCTTGG 3′	298	58	30
m EMMPRIN[a]	5′ GTCTAGAGGATCCATCCAAACCTCGGTCCA 3′	5′ TCTAGAGAATTCCACGCAGTGAGATGGTTT 3′	542	54	30
h GAPDH[a]	5′ GGAGTCAACGGATTTGGT 3′	5′ GTGATGGGATTTCCATTGAT 3′	206	55	30
m 36B4	5′ TCATTGTGGGAGCAGACA 3′	5′ TCCTCCGACTCTTCCTTT 3′	832	54	25
h EMMPRIN[b]	5′ GCACAGTCTTCACTACCGTA 3′	5′ CCTCCATGTTCAGGTTCTCA 3′	442	57	

[a]h is for human, m is for mouse.
[b]Those h EMMPRIN primer pairs were performed in the GCT tumor cell cultures.

magnesium chloride and sterile water in a total volume of 25 μl. The house keeping gene, human GAPDH or mouse 36B4, were used as internal controls. The reaction was run in a DNA thermal cycler (model 2400, Perkin-Elmer) from 25 to 30 cycles of 40 s of denaturation at 94°C, 40 s of annealing at individual optimal temperature, 40 s or 1 min of extension at 72°C, with 10 min of extra extension used for the last cycle. PCR products were identified in a 1.2% modified agarose gel (Promega, USA) and stained with ethidium bromide (EtBr). Densitometry was used to estimate the intensity of PCR products.

In-Situ Hybridization

To investigate the localization of EMMPRIN mRNA in GCT tissues, we performed ISH as previously described by Zheng et al. [1993] using antisense EMMPRIN riboprobe. The diogoxigenin (DIG)-labeled antisense EMMPRIN riboprobe was prepared using a DIG RNA labeling kit (Roche). A human EMMPRIN cDNA was obtained by PCR and was subcloned into the pGEM^{TM}-T Easy vector (Promega, Sydney, Australia). The plasmid was linearized with *Nco I* and reversed transcription by SP6 RNA polymerase to generate a 0.35 kb-long antisense probe at a concentration of 10 ng/ml. GCT tissues were imprinted with minimal pressure onto Rnase-free silianized glass slides. The imprint slides were stored at −20°C.

After fixation with 4% Paraformaldehyde (Merck, Darmstadt, Germany) in phospate buffered saline (PBS) for 30 min at 4°C, GCT imprint specimens were rinsed in 0.2% Triton X in PBS for 5 min. Cells on the slides were incubated with 2 μg/ml of proteinase K in 0.1 M Tris buffer (pH 8.0) and 50 mM EDTA (pH 8.0) at 37°C for 20 min in a humidified chamber, followed by 0.1% glycine in PBS for 2 min briefly. The slides were post fixed in 4% Paraformaldehyde in PBS for 15 min at 4°C and were then treated with Rnase-free DNase (1 U/ml) for 30 min at 37°C to ensure specificity of mRNA hybridization. Slides were washed in PBS between each treatment. Before hybridization, the slides were incubated with 100 μg/ml of denatured salmon sperm DNA in prehybridization buffer (50% (v/v) formamide, 5 × SSC, DEPC treated-water) at 37°C for 1 h. Hybridization was performed at 37°C for at least 16 h in a moist chamber with 50 μl of ISH solution (50% deionised formamide, 10% Dextran sulfate, 1 mg/ml denatured salmon sperm DNA), 5 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and DIG-labeled RNA riboprobe against human EMMPRIN at the final concentration of ~0.5 ng/ml on each sections.

After hybridization, the slides were rinsed twice in 2 × SSC, twice in 1 × SSC, and finally twice in 0.1 × SSC at 37°C for 15 min and then washed 1% of blocking solution in PBS for 30 min at room temperature. Detection of hybridized probes was visualized with the aid of a riboprobe detection kit according to manufacturer's instruction (Boehringer Mannheim, Germany).

Immunohistochemistry

To further confirm the cellular localization of EMMPRIN in GCT, immunohistochemical staining was performed as previously described by Zheng et al. [1998] with minor modifications. GCT imprints were fixed with acetone and were blocked for endogenous peroxidase activity by incubation in 0.3% H_2O_2 for 10 min, washed in PBST (0.1% TritionX-100, PBS), and then treated with 20% normal horse serum for 30 min before incubation overnight at 37°C with a 1:100 dilution of mAb 8G6 or mAb CD68 (1:50). Slides were then rinsed for three times in PBST, incubated for 1 h at 37°C with a 1:250 dilution of secondary anti-mouse immunoglobulin (IgG) (Sigma, Germany), followed by three washes in PBST and color developed for 5 to 30 min in a moist chamber at room temperature using a liquid 3,3′-diaminobenzidine (DAB) substrate-chromogen solution kit (DAKO, USA). Slides were counterstained in hematoxylin, dehydrated in graded ethyl alcohol (70%, 90%, 100%), and mounted in Depex. Preparation in which the primary antibody to mAb 8G6 was omitted served as negative controls.

Culture of Stromal-Like Tumor Cells of GCT

Two samples of GCT solid tumors were freshly collected and chopped in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin as previously described [Zheng and Fan, 1994]. The resultant cell suspension together with small pieces of tissues were then transferred to 25-cm^2 flasks for culture at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Half of the culture media was changed every 3 days with above fresh DMEM. Upon reaching confluence, primary cultures were sub-cultured.

Total cellular RNA from the cultured cells at various passages, U2OS cell line and normal human fibroblast were subjected to RT-PCR analysis for the mRNA expression of EMMPRIN.

Regulation of EMMPRIN in $RAW_{264.7}$ Macrophage

Mouse $RAW_{264.7}$ cells were sub-cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 10% L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. To analyze the effect of RANKL on regulation of EMMPRIN expression during osteoclastogenesis, mouse $RAW_{264.7}$ cells were seeded in a 6-well plate to a density of 5×10^4 cell/well, and treated with GST-rRANKL (100 ng/ml) [Xu et al., 2000], for different time points from 0 to 5 days. Total RNA was extracted from these cells using the RNAqueous™ Kit (Ambion Inc., Austin, TX). RT-PCR was performed as described previously to examine the EMMPRIN mRNA expression.

The cycle-dependent PCR was performed to amplify EMMPRIN mRNA in $RAW_{264.7}$ cells to establish a linear amplification curve. Then, 30 cycles of PCR was chosen for the semi-quantification of EMMPRIN mRNA expression. The mRNA levels of EMMPRIN in different treatment groups were normalized to mouse 36B4 mRNA as an internal standard.

Densitometry and Statistical Analysis

The relative intensity of PCR products was measured with the ScnImage densitometry software (Scion Corp., Frederick, MD). The significance of the gene expression of EMMPRIN, MMP-1, 2 and TIMP-1, 2 were determined using Student's *t*-test. Results were considered statistically significant at *P* values less than 0.05.

RESULTS

Gene Expression of EMMPRIN mRNA in GCT

We investigated the mRNA expression of EMMPRIN in GCT tissues. As shown in Figure 1, there were five cases in stage III (lanes: 1, 2, 3, 5, 11), four cases in stage I (lanes: 4, 7, 8, 9), three cases in stage I/II (lanes: 15, 16, 17) and five in stage II (lanes: 6, 10, 12, 13, 14) examined. The result demonstrated that EMMPRIN mRNA expression was detected at various levels with stage I & II and stage III (Fig. 1A). Comparison of the ratio of EMMPRIN to house keeping gene, showed a significant

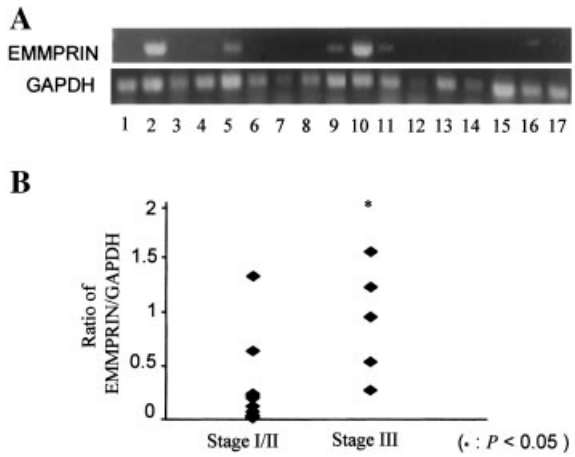

Fig. 1. EMMPRIN gene expression in GCT. **A**: Semi-quantitative RT-PCR of EMMPRIN in GCT. **Lanes 1, 2, 3, 5, 11** were stage III of GCT. **Lanes 6, 10, 12, 13, 14** were stage II of GCT. **Lanes 15, 16, 17** were stage I of GCT. GAPDH, a housekeeping gene was used as a control. **B**: Correlation of EMMPRIN and clinical stage of GCT. The level of EMMPRIN expression was expressed as the ratio of EMMPRIN/GAPDH. Note that the level of EMMPRIN mRNA is significantly different ($P < 0.05$) between stage I & II and stage III of GCT.

($P < 0.05$) difference of EMMPRIN expression between stages I & II and stage III. The levels of EMMPRIN in Enneking's clinical stage III of GCT were significantly greater than that of stage I & II, indicating that there was significant correlation of EMMPRIN expression and the Enneking's Clinical Stage of GCT at presentation (Fig. 1B).

Gene Expression of MMP-1, 2 and TIMP-1, 2 in GCT

As EMMPRIN is considered to be a key regulator for the production of MMPs, we next examined the mRNA expression of MMP-1, 2 and TIMP-1, 2 in GCT, we performed RT-PCR in a series of 11 cases, including nine in stage I/II (lanes: 2–8, 10, 11) and two in stage III (lanes: 1, 9). As shown in Figure 2, the results revealed that MMP-1, and TIMP-1, 2 mRNA were abundantly expressed in all the cases, however, MMP-2 mRNA was relatively less abundant. Comparison of the ratio of MMP-1, 2, and TIMP-1, 2 to GAPDH, (indicated that) there was no significant correlation between the clinical stages of GCT and the expression level of MMPs and TIMPs.

Localization of EMMPRIN in GCT

To determine the localization of EMMPRIN at cells of GCT, ISH, and IH were performed on

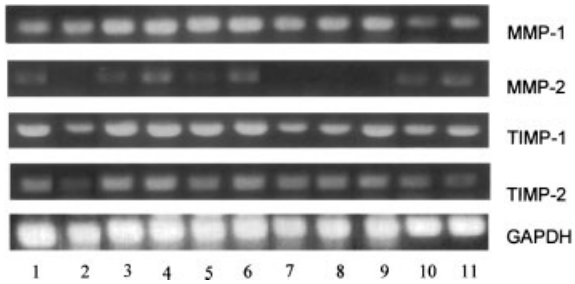

Fig. 2. Gene expression of MMP-1, 2, and TIMP-1, 2 in GCT as detected by semi-quantitative RT-PCR. **Lanes 2–8, 10, 11** were stage I and II GCT. **Lane 1** and **9** were stage III of GCT.

GCT imprints using DIG-labeled specific antisense EMMPRIN riboprobe and mAb 8G6 against human EMMPRIN, respectively. A total of 8 cases were examined in this study. Both ISH and IH demonstrated that EMMPRIN expression is present abundantly at the multinuclear osteoclast-like giant cells of GCT (Figs. 3B,C, 4A,B,C). IH staining showed strong positive signals on the cell membrane of giant cells. Interestingly, both macrophage-like mononuclear cells (Figs. 3B, 4B) and spindle-shaped stromal tumor cells (Fig. 3C, 4B) were also positive but the staining intensity is weaker. Treatment with anti-mouse immunoglobulin showed no staining on GCT imprints,

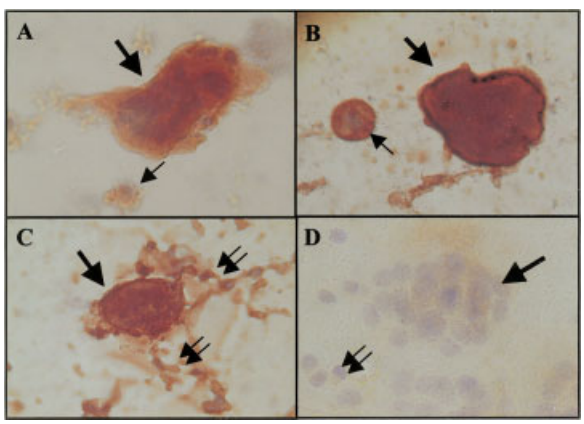

Fig. 3. Immunohistochemistry of EMMPRIN in GCT. **A**: Multinuclear osteoclast-like giant cell (big arrow) and round-shaped macrophage-like cells (small arrow) were positive for CD68. **B**: Multinuclear osteoclast-like giant cell (big arrow) macrophage-like cells and spindle shaped (small arrow), were positive for EMMPRIN in a stage III GCT sample. Note that the staining was on the cytoplasmic membrane of the cells. **C**: Multinuclear osteoclast-like giant cells (big arrow), and spindle shaped stromal-like tumor cells (small arrow) were positive for EMMPRIN in a stage I GCT sample. Note that staining was mainly on the cytoplasmic membrane of the cells. **D**: Negative control of GCT without primary antibody (Magnification 450×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

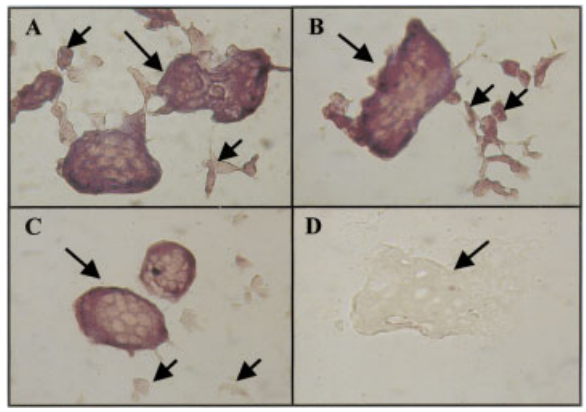

Fig. 4. In-situ hybridization of EMMPRIN. Multinuclear osteoclast-like giant cells (big arrow) and mononuclear cells including round-shaped macrophage-like cells and stromal-like cells (small arrow) were expressed EMMPRIN mRNA in cytoplasm of a stage III GCT sample (**A**), a stage II GCT sample (**B**), a stage I GCT sample (**C**). Negative control (**D**) was used by treatment of RNase before the FISH as described in Materials and Methods. Note that the signal of EMMPRIN in multinuclear osteoclast-like cells seems to be stronger than the mononuclear cells (Magnification 450×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

indicating that mAb 8G6 was specifically immunoreacted with EMMPRIN (Fig. 3D). Furthermore, treatment with RNase resulted in a loss of signals in all cells on negative controls, indicating the specificity of EMMPRIN mRNA hybridizing with the labeled antisense EMMPRIN riboprobe (Fig. 4D). Similar results were obtained in three-independent experiments.

EMMPRIN Expression in Stromal-Like Tumor Cell Culture

As shown by both IH and ISH, the stromal-like tumor cells were weakly stained with EMMPRIN. To confirm the mRNA expression of EMMPRIN in cultured stromal-like tumor cells of GCT, late passage cultured cells which contain stromal-like tumor cells but not multinucleated giant cells (>8–9th) were examined [Huang et al., 2000]. The results showed that EMMPRIN gene transcripts were abundantly present in cultured stromal tumor cells of GCT at either 8th (P8) or 11th (P11) passage (Fig. 5), indicating that tumor cells express EMMPRIN in GCT tissue cultures.

The Effect of RANKL on EMMPRIN Regulation in $RAW_{264.7}$ Cells

Given RANKL is one of the key cytokines produced by stromal-like tumor cell of GCT for the recruitment of macrophage and generation

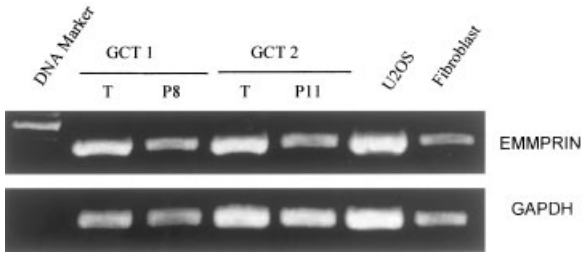

Fig. 5. Expression of EMMPRIN mRNA in stromal-like tumor cells derived from GCT cultures. Note that EMMPRIN mRNA was detected at the eighth (P8) and eleventh (P11) passages in two cases of GCT compared with the original tumor tissues (T). Human osteosarcoma cells (U2OS) and normal human fibroblasts were used as controls.

of osteoclast lineages, [Huang et al., 2000] we further examined the effect of RANKL on the regulation of EMMPRIN mRNA expression in mouse macrophages. Mouse $RAW_{264.7}$ cells, a macrophage-cells line, have been shown to be capable of differentiating into osteoclast-like cells in the presence of RANKL [Huang et al., 2000; Xu et al., 2000]. We investigated whether RANKL has the ability to influence EMMPRIN gene expression during osteoclastogenesis in vitro. The cycle-dependent PCR was performed to amplify EMMPRIN mRNA in $RAW_{264.7}$ cells (Fig. 6A). Quantification of the mRNA level of EMMPRIN at different time points, from day 0 to day 5, indicated that RANKL was able to up-regulate EMMPRIN mRNA in $RAW_{264.7}$ cells during osteoclastogenesis (Fig. 6B). These data suggest that the production of EMMPRIN in osteoclast-like giant cells may be regulated by stromal-like tumor cells-derived RANKL.

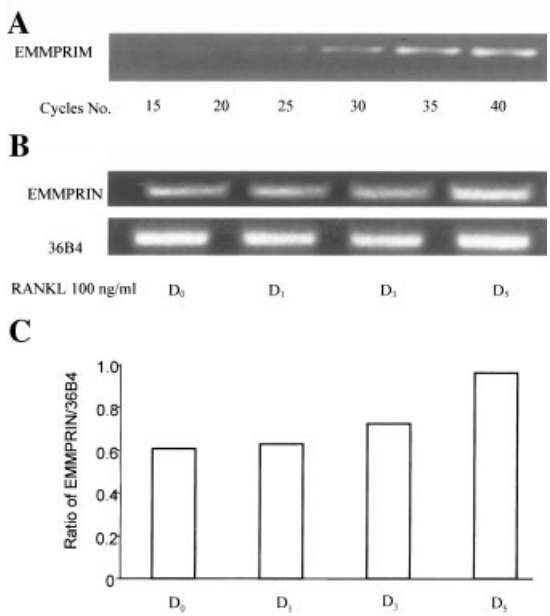

Fig. 6. Regulation of EMMPRIN in $RAW_{264.7}$ cells by RANKL. **A**: To establish a linear amplification curve, cycle-dependent PCR was carried out to amplify mouse EMMPRIN mRNA in $RAW_{264.7}$ cells in the absence of RANKL. **B**: Expression of EMMPRIN mRNA in $RAW_{264.7}$ treated with RANKL. Thirty cycles of PCR were used to achieve the quantitation. D0, No RANKL treatment, D1, RANKL treatment for 1 day, D3, RANKL treatment for 3 days, D5, RANKL treatment for 5 days. The results indicated that EMMPRIN mRNA was up-regulated during osteoclastogenesis by RANKL in vitro. PCR products were analyzed on a 1.2% agarose gel. **C**: The level of EMMPRIN expression was expressed as the ratio of EMMPRIN/GAPDH following the densitometry measurement of PCR products.

DISCUSSION

Previous studies have shown that EMMPRIN was expressed by human carcinoma cells [Kataoka et al., 1993; Guo et al., 1997]. EMMPRIN was capable of stimulating fibroblasts to produce the expression of MMP-1, MMP-2, and MMP-3 in tumor cells and Chinese hamster ovary cells [Kataoka et al., 1993; Guo et al., 1997]. It has been suggested that the expression of EMMPRIN is associated with tumor aggressiveness prompted by production of MMPs by stromal-like cells or fibroblasts [Dalberg and Eriksson, 2000; Zucker and Cao, 2000]. In the present study, we have shown for the first time the expression of EMMPRIN in the solid tumor of GCT. By RT-PCR analysis, we showed that the ratio of EMMPRIN to GAPDH was significantly higher in stage III GCTs when compared with stage I/II GCTs ($P < 0.0104$). The expression of EMMPRIN seems to be greatly correlated with the Enneking's Clinical Classification of GCT. Our studies suggest EMMPRIN may be considered to be one of the biological factors for the progression of GCT tumor. Several biological predictors for the aggressiveness of GCT have been reported. The overexpression of the *c-myc* oncogene distinctly correlates with occurrence of metastasis to the lung in GCT. However, the prognostic relevancy of *c-fos* oncogene seems to be minor in the tumorigenesis of this neoplasm [Gamberi et al., 1998]. We have also reported a strong correlation ($P < 0.0357$) between the overexpression of vascular endothelial growth factors (VEGF) and the advanced stage (stage III) of GCT [Zheng and Xu, 2000].

EMMPRIN has been shown to be present on the surface of the neoplastic cells, but not on

fibroblasts [Muraoka et al., 1993; Zucker and Biswas, 1994; van den Oord and Paemen, 1997]. In GCT, we have demonstrated, by using both IH and ISH, that EMMPRIN was intensively present in the multinucleated osteoclast-like giant cells, but less intensive in the spindle-shaped stromal-like tumor cells. However, by RT-PCR, stromal-like tumor cells derived from GCT culture express EMMPRIN mRNA. Note that the stromal-like tumor cells are the putative neoplastic components of GCT, whereas the multinucleated giant cells are likely the reactive elements [Zheng et al., 1998]. It has been shown that neoplastic cells of GCT are capable of producing transforming growth factor-beta 1 (TGF-β1), [Zheng and Fan, 1994] urokinase-type plasminogen activator (U-PAR), [Zheng et al., 1995] monocyte chemoattractant protein-1 (MCP-1), [Zheng et al., 1998] VEGF, [Zheng and Xu, 2000], and RANK, [Huang et al., 2000] to stimulate recruitment of reactive osteoclast-like giant cells and macrophages. It is not clear why osteoclast-like giant cells, such a non-neoplastic element, express abundant EMMPRIN, as most other normal adult tissues, including epidermis, retinal pigment epithelium, breast lobules, and ductules, present a very low level of EMMPRIN expression [DeCastro et al., 1996; Marmorstein et al., 1996]. However, the fact that normal macrophages, [Liang et al., 2002; Major et al., 2002] also produce EMMPRIN may reflect that monocyte-osteoclastic linage cells are the candidates for EMMPRIN production. Interestingly, RANKL, which can be produced by stromal-like tumor cells of GCT, regulates gene expression of EMMPRIN in RAW-cell derived osteoclasts. Thus, our data suggests that EMMPRIN production in both stromal-like tumor cells and osteoclast-like giant cells are associated with aggressiveness of GCT.

Although the specific function of MMPs remains unknown, a number of studies have determined that MMPs were synthesized in tumor cells and adjacent stromal-like cells to regulate tumor invasion, and metastasis [Rao et al., 1997; Dalberg and Eriksson, 2000; Zucker and Cao, 2000]. MMP-2 and MMP-9 were localized in the mononuclear stromal tumor cells in late-passage cultures, and may play an important role in regulating tumor behaviors of GCT [Ueda et al., 1996; Rao et al., 1997; Teti et al., 1998]. The production of MMP-9 in GCT has also been reported to accelerate the extracellular matrix damage associated with lung metastasis, vascular invasion and recurrent malignancy of the conventional GCTs [Ueda et al., 1996; Masui and Ushigome, 1998; Oda and Sakamoto, 2001]. The expression and activation of MMP-1 and MMP-3 stimulated by EMMPRIN have been demonstrated to correlate with the synovium invasion in rheumatoid arthritis [Tomita and Nakase, 2002]. However, by RT-PCR analysis, we showed that there is no correlation between the expression levels of MMP-1, 2 and TIMP-1, 2 with the Enneking's Clinical Stages of GCT. Bearing in mind that there is still a limitation of small numbers of cases, and also other MMPs molecules have not been examined, the potential prognostic value of MMPs expression in GCT is still uncertain.

In the present study, we have shown that RANKL up-regulated the mRNA level of EMMPRIN in mouse macrophages, the $RAW_{264.7}$ cells, during osteoclastogenesis in vitro. EMMPRIN was expressed in both osteoclast-like giant cells and stromal-like tumor cells of GCT. The level of EMMPRIN expression is associated with Enneking's Clinical Stage of Classification. In conclusion, EMMPRIN might be a regulatory factor involved in MMP-dependent tumor behaviors of GCT.

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