Relation between Histological Intensity of Transforming Growth Factor- β Isoforms in Human Osteosarcoma and the Rate of Lung Metastasis

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*Relation between Histological Intensity of Transforming Growth Factor- β Isoforms
in Human Osteosarcoma and the Rate of Lung Metastasis.* Tohoku J. Exp. Med.,
1998, **184** (2), 133–142 ——— Eventhough adjuvant chemotherapy has improved the
5-year survival rate of osteosarcoma patients, a significant percentage of patients
eventually die from lung metastasis. Since transforming growth factor- β (TGF- β)
has been demonstrated to be related to the tumor progression, we investigated the
clinical implications of the presence of TGF- β isoforms in 16 human osteosarcoma
tissue. There were 10 males and 6 females with a mean age of 20.8 years of age
(range, 8 to 57 years). Biopsied specimen before chemotherapy was fixed in 10%
formalin, demineralized and followed by paraffin embedding. The locations of
tumor included femur (10), tibia (3), humerus (1), fibula (1), and ilium (1).
Histologic subtypes included osteoblastic (11), chondroblastic (2), and fibroblastic
(3). All patients were followed for a minimum of 1 year (range 12 to 44 months)
or to the development of lung metastasis. Five patients (31.3%) developed
subsequent lung metastasis during the follow up. We used immunohistochemistry
technique to investigate the presence of the TGF- β isoforms in osteosarcoma tissue
and its relationship to the subsequent pulmonary metastasis. The results showed
the presence of one or more TGF- β isoforms in tumor cells in osteosarcoma tissues
(13 of 16, 81.3%) in all of the subtypes. However, minimal presence of TGF- β
isoforms was shown in the tumor bone matrix. The expression of TGF- β 1 or
TGF- β 3 isoforms was associated with a higher rate of subsequent lung metastasis
($p < 0.05$, chi-square test). Further research is warranted to determine the utility
of routine TGF- β analysis in the clinical practice. ——— TGF- β ; osteosarcoma;
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Transforming growth factor- β (TGF- β) is a local growth factor which is
presented in normal tissues, especially platelets and bone matrix, as well as

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transformed cells (Roberts et al. 1981; Childs et al. 1982; Assoian et al. 1983; Seyedin et al. 1986; Robey et al. 1987; Sporn et al. 1987). In addition to regulation of physiological processes, TGF- β 1 has been shown to be involved in many pathological conditions, such as inflammation, tumor formation and progression (Roberts et al. 1988; Noda and Rodan 1989; Santala and Heino 1991; Massague et al. 1992; Mooradian et al. 1992; Kim and Ballock 1993; Newman 1993; van Waes 1995). Various kinds of tumor cells have been proved to synthesize TGF- β , including sarcoma, squamous cell carcinoma, lung cancer, pancreatic cancer, breast cancer, hepatoma, renal cancer, adenocarcinoma (gastric and colon cancer), lymphoma, and myeloma (Sporn et al. 1987; Daughaday and Deuel 1991). Recently its presence has been elucidated in mouse embryo and human osteosarcoma tissues (Pelton et al. 1991; Kolen et al. 1996). TGF- β s are shown to be related to the formation of tumor matrix as well as neoangiogenesis (Heino and Massague 1989; Daughaday and Deuel 1991; Kim and Ballock 1993; Newman 1993; Huang and Chakrabarty 1994), thus they may play an important role in the tumor metastatic cascade and influence the clinical outcomes. However, the presence of TGF- β isoforms in the osteosarcoma tissue to its clinical outcomes remains unsolved up to the present.

Local surgery alone yielded 5-year survival rates of 10–20% for patients with osteosarcoma. The current 5-year survival rates of 50–80% are due mainly to the use of neoadjuvant chemotherapeutic protocols (Bacci et al. 1991; Glasser et al. 1992). Yet, a significant number of patients remain poor or nonresponsive and develop subsequent pulmonary metastasis. Recent studies have shown that osteoblasts and osteosarcomas are capable of producing TGF- β isoforms (Robey et al. 1987; Kolen et al. 1996).

The antibodies to TGF- β isoforms permits an easy and simple immunohistochemical procedure for rapid determination of TGF- β isoforms in tumor tissue specimens. This report details the immunolocalization of TGF- β isoforms in 16 human osteosarcoma biopsy specimens using monoclonal antibodies against TGF- β 1, TGF- β 2, and TGF- β 3 isoforms. Since the TGF- β has been reported to be related to the formation of tumor matrix and neoangiogenesis, the hypothesis to be tested in this study is that TGF- β isoforms expression in prechemotherapy osteosarcoma cells correlates with the rate of subsequent pulmonary metastasis. The medical oncologist might use the presence of TGF- β isoforms in a tumor biopsy specimen to tailor a more aggressive treatment protocol.

MATERIALS AND METHODS

Sixteen osteosarcoma before chemotherapy was biopsied and fixed in 10% formalin. Then specimens were demineralized by Decalcifier solution (Life Science Int. Ltd., Cheshire, UK) for 2–3 days at room temperature and embedded in paraffin wax after dehydration through graded alcohol. There were 10 males and 6 females with a mean age of 20.8 years (range, 8 to 57 years). The locations

TABLE 1. *TGF- β isoforms in histological subtypes of osteosarcoma*

Type	Total	TGF- β isoforms (-)	TGF- β isoforms (+)	Chi-square test
TFG- β 1				
Osteoblastic	11	5	6 (54.6%)	n.s.
Fibroblastic	3	3	0 (0%)	
Chondroblastic	2	1	1 (50%)	
Total	16	9	7 (43.8%)	
TGF- β 2				
Osteoblastic	11	5	6 (54.6%)	n.s.
Fibroblastic	3	0	3 (100%)	
Chondroblastic	2	0	2 (100%)	
Total	16	5	11 (68.8%)	
TGF- β 3				
Osteoblastic	11	4	7 (63.6%)	n.s.
Fibroblastic	3	2	1 (33.3%)	
Chondroblastic	2	0	2 (100%)	
Total	16	6	10 (62.5%)	

n.s., not significant.

of the tumor included femur (10), tibia (3), humerus (1), fibula (1), and ilium (1). Histologic subtypes included osteoblastic (11), chondroblastic (2), and fibroblastic (3) (Table 1). After biopsy and diagnosis, patients received the neoadjuvant chemotherapy consisting mainly of adriamycin, methotrexate, cis-platinum, and ifosfamide. Subsequently, patients underwent limb salvage surgery or an amputation. All patients received subsequent chemotherapy followed by surgical resection or amputation. Pathology reports and all histology of these specimens were reviewed and the percentage of necrosis was confirmed on regular staining of hematoxylin and eosin (H & E). All patients were followed for a minimum of 1 year (range 12 to 44 months) or to the development of lung metastasis. Five patients (31.3%) developed subsequent lung metastasis during the follow-up.

For immunohistochemical study, sections were bathed in 0.1% protease (Sigma Chemical Co., St. Louis, MO, USA)/PBS (pH 7.4) for 15 minutes at 37°C and then washed in PBS (pH 7.4) for antigen retrieval. Sections were treated with 3% H₂O₂ for 30 minutes at room temperature to block endogenous peroxidase activity. Slides were incubated with the TGF- β isoforms antibody (1 μ g/ml, Santa Cruz Biotech, CA, USA) for one hour at room temperature in a humidity chamber. Antibody to each TGF- β 1 isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, is the monoclonal antibody found to specifically react against each isoform of TGF- β without cross reactivity to other isoforms. The specificity of antibody has been demonstrated by Western immunoblotting, immunoprecipitation and im-

Fig. 1. Immunohistochemical localization of the TGF- β isoforms in human osteosarcoma with monoclonal antibodies against TGF- β isoforms, i.e., TGF- β 1, TGF- β 2, and TGF- β 3.

A: Focal positive staining for TGF- β 1 of the tumor cells in the tumor tissue from a 17 years old male having the chondroblastic type osteosarcoma (arrow). The chondroblastic area in this case was negative for staining (66 $\times$).

B: Tumor tissue from a 21 year-old male having an osteoblastic osteosarcoma in the proximal tibia. Both the endothelial cells (arrowheads) and the tumor cells were stained positively for TGF- β 1 (66 $\times$).

Fig. 1. C: Tumor tissue from a 15 year-old male having an osteoblastic osteosarcoma in the distal femur. TGF- β 2 was demonstrated in tumor cells as well as multinucleated osteoclast type giant cells (arrows) ($66\times$). D: Diffuse staining for TGF- β 3 on the tumor cells in the tumor tissue from a 13 years old male having an osteoblastic osteosarcoma ($66\times$). The matrix of chondromatous matrix showed negative reaction to TGF- β 3 staining, whereas peripheral and surrounding area had positive reaction (arrows).

TABLE 2. *TGF- β isoforms localization and the occurrence of subsequent lung metastasis*

Type	Total	Lung metastasis (–)	Lung metastasis (+)	Chi-square test
TGF- β 1 (–)	9	8	1 (11.1%)	<0.05
TGF- β 1 (+)	7	3	4 (57.1%)	
TGF- β 2 (–)	5	4	1 (20%)	n.s.
TGF- β 2 (+)	11	7	4 (36.4%)	
TGF- β 3 (–)	6	6	0 (0%)	<0.05
TGF- β 3 (+)	10	5	5 (50%)	

n.s., not significant.

munohistochemistry techniques. After primary antibody incubation, slides were washed with PBS and then incubated with link antibody (Dako Ltd., High Wycombe, UK) for 15 minutes at room temperature in a humidity chamber. Then, sections were incubated with streptavidin-biotin complex (Dako Ltd.) for 15 minutes at room temperature in a humidity chamber. Finally, 3-3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) solution (containing 0.024% H_2O_2 and 0.067% DAB in PBS, pH 7.4) was applied. Sections were counterstained with Harris' hematoxylin, dehydrated in graded ethanol, and sealed with coverslip for final histologic analysis. For negative control, certain osteosarcoma specimens were incubated with isotype-matched mouse IgG (1 $\mu\text{g}/\text{ml}$) for two hours at room temperature in place of primary antibody. All sections and controls were evaluated by two independent observers to confirm the presence of TGF- β isoforms in the tumor tissue and controls. The correlation between the expression of TGF- β isoforms was then made with the histologic subtypes and the rate of pulmonary metastasis. Chi-square test was used to evaluate the statistical significance which was determined to have a $p < 0.05$.

RESULTS

Osteosarcoma specimens treated with mouse IgG in place of the primary antibody demonstrated no staining. Strong brown staining was demonstrable in the cytoplasm of tumor cells from all subtypes of osteosarcomas indicating the presence of various TGF- β isoforms (Fig. 1). Either focal positive staining (Fig. 1A) or diffuse staining (Fig. 1B-1D) for TGF- β isoforms in the cytoplasm of the tumor cells has been demonstrated in the osteosarcoma tumor tissue. Moderate to intense staining of endothelial and perivascular muscle cells (Fig. 1B) were also shown in osteosarcoma tissue specimen for TGF- β isoforms. In addition, multinucleated osteoclast type giant cells in the tumor tissue also showed strong staining for all TGF- β isoforms (Fig. 1C). On the other hand, minimal presence of TGF- β isoforms was demonstrated in the tumor bone matrix. No stain was

found in the mineralized bone and chondromatous zone (Fig. 1A and 1D). Either TGF- β 1, TGF- β 2, or TGF- β 3 isoform was found in the histological subtypes of osteosarcoma (Table 1). The TGF- β 1, TGF- β 2, and TGF- β 3 staining in tumor cells was not related to the histological subtypes by chi-square test. In addition, the results demonstrated the presence of one or more TGF- β isoforms in tumor cells in the osteosarcoma specimens (13 of 16, 81.3%), for TGF- β 1, 7 of 16 (43.8%); TGF- β 2, 11 of 16 (68.8%); and TGF- β 3, 10 of 16 (62.5%).

Five patients developed lung metastasis in the current follow-up. Four of 7 with positive expression of TGF- β 1 (57.1%) in tumor cells and 1 of 9 (11.1%) with negative TGF- β expression developed subsequent lung metastasis during follow-up; so did 4 of 11 patients (36.4%) with positive TGF- β 2 as compared to 1 of 5 patients with negative TGF- β 2 immunoreactivity in the tumor cells; and 5 of 10 patients (50%) with positive TGF- β 3 as compared to none of 6 patients (0%) with negative TGF- β 3 immunoreactivity in the tumor cells. The frequency of lung metastasis in the patient with positive TGF- β 1 and TGF- β 3 staining in the cells was statistically higher than that of TGF- β 1 and TGF- β 3 negative patients for development of subsequent lung metastasis ($p < 0.05$, Table 2).

DISCUSSION

This study demonstrated the immunolocalization of TGF- β 1, TGF- β 2, and TGF- β 3 in tumor cells in the specimens of 16 patients with variants of osteosarcoma, including osteoblastic, chondroblastic, and fibroblastic subtypes. TGF- β isoforms were shown in both the tumor cells, as well as in the endothelial cells and perivascular muscle cells. The expression of TGF- β 1 and TGF- β 3 in the tumor cells was related to a higher rate of subsequent lung metastasis for expression of any isoforms of TGF- β .

In this study, monoclonal antibodies against TGF- β 1, TGF- β 2, and TGF- β 3 were used to detect these proteins in 16 osteosarcoma biopsies taken before application of chemotherapy. Samples were chosen to provide the maximum percentage of viable cells, allowing an accurate assessment of the tumor's TGF- β 1, TGF- β 2, and TGF- β 3-producing capability. The results were graded according to the percentage of cells in the tissue which stained positively for TGF- β 1, TGF- β 2, and TGF- β 3. The immunohistochemical techniques were utilized for the evaluation of TGF- β 1, TGF- β 2, and TGF- β 3 expression in the prechemotherapy biopsy specimens.

Surgery and neoadjuvant chemotherapy remain the main treatment modality for osteosarcoma, but a number of patients appear to be poor or nonresponders based on the subsequent disease progression (Bacci et al. 1991; Glasser et al. 1992). With regard to the tumor progression, TGF- β 1 has been demonstrated to be involved in both the formation of tumor matrix and neoangiogenesis (Heino and Massague 1989; Daughaday and Deuel 1991; Mooradian et al. 1992; Kim and Ballock 1993; Huang and Chakrabarty 1994; van Waes 1995). TGF- β 1 has been

proven to regulate the expression of adhesion molecule and integrin receptors on the tumor cells, thus affecting tumor cell adhesion and metastatic potential (Santala and Heino 1991; Huang and Chakrabarty 1994). Therefore, investigation of the expression of TGF- β isoforms in the tumor tissue may provide important information to the clinical oncologist. This study demonstrated that TGF- β isoforms expression in the biopsy specimens may allow oncologists to predict those osteosarcomas that will have a poor response to modern therapy. Physicians could then alter protocols to treat these probably more resistant tumors. These results necessitates a prospective study.

Several studies have reported the presence of TGF- β 1, TGF- β 2 and TGF- β 3 in osteosarcoma tissue, but the percentage of positive TGF- β 1, TGF- β 2 and TGF- β 3 expression in the groups of samples studied varies widely. Using immunohistochemical methods with a monoclonal antibodies, Kolen et al. (1996) found that majority of osteosarcoma tissues (96%) produced one or more TGF- β isoforms, generally TGF- β 1 and TGF- β 3 than TGF- β 2. They also demonstrated strong staining of osteoclasts involved in the bone resorption. The results in our study concurred with the aforementioned report. However, the percentage of positivity was less than that of the previous study. In the current study, we utilized the immunohistochemical methods to demonstrate that TGF- β 1, TGF- β 2, and TGF- β 3 can be present in the cytoplasm of malignant cells with primitive mesenchymal features i.e., undifferentiated spindle cells. Furthermore, we demonstrated the presence of TGF- β 1, TGF- β 2, and TGF- β 3 in osteoclast-like giant cells in the tumors.

Using immunohistochemical methods with a monoclonal antibodies, Kolen et al. (1996) found that majority of osteosarcoma tissues expressed multiple small vessels with moderate to intense staining of endothelial and perivascular muscle cells for TGF- β 1 (70%), TGF- β 2 (64%), and TGF- β 3 (70%). The tumorous perivascular smooth muscles cells and endothelial cells were stained intensely in the current study. The expression of TGF- β isoforms may be related to the angiogenetic activity.

Although Kolen et al. (1996) claimed that osteosarcoma with TGF- β 1, TGF- β 2, and TGF- β 3 had no statistically significant higher rate of lung metastasis, we found out a positive correlation between TGF- β 1 and TGF- β 3 immunostaining and subsequent lung metastasis. However, there are some limitations for this study. The small sample size precluded a multivariate analysis study. In addition, the osteosarcoma is composed of heterogeneous cell population, the biopsy specimen may not be representative of the whole tumor. Further multi-institutional prospective study is needed for investigation of their clinical implication.

In conclusion, TGF- β 1, TGF- β 2, and TGF- β 3 expression was demonstrated in the osteosarcoma tissue. The presence of this protein prepared from paraffin section was associated with a higher rate of subsequent lung metastasis. How-

ever, the small sample size precluded any conclusion regarding ultimate prognosis thus requiring the need for a further large scale investigation.

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