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Concomitant tumour resistance in patients with osteosarcoma

A CLUE TO A NEW THERAPEUTIC STRATEGY

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Concomitant tumour resistance (CTR) is a unique phenomenon in which animals harbouring large primary tumours are resistant to the growth of smaller metastatic tumours by systemic angiogenic suppression. To examine this clinically, in ten patients with osteosarcoma, we investigated the effects of removal of the primary tumour on the development of pulmonary metastases, the systemic angiogenesis-inducing ability and the serum levels of several angiogenesis modulators.

We found that removal of the primary tumour significantly elevated systemic angiogenesis-inducing ability in five patients who had post-operative recurrence of the tumour. Post-operative elevation of the angiogenesis-induced ability was suppressed by the addition of an angiogenic inhibitor, endostatin. Also, primary removal of the tumour decreased the serum levels of vascular endothelial growth factor and endostatin.

These findings suggest, for the first time, the presence of CTR in patients with osteosarcoma for whom post-operative antiangiogenic therapy may be used to prevent the post-operative progression of micrometastases.

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Osteosarcoma is the most common primary malignant bone tumour in adolescents and young adults. Treatment consists of a combination of chemotherapy and resection of the tumour.[1-4] Although this therapeutic strategy has improved the prognosis for many patients with osteosarcoma, some still die from post-operative pulmonary metastases.[1] Therefore, clarification of the mechanism of the post-operative progression of pulmonary metastases is necessary.

Concomitant tumour resistance (CTR) is a unique phenomenon in which animals harbouring large primary tumours are resistant to the growth of smaller metastatic tumours.[5] Until recently the underlying molecular mechanisms have been largely unknown but studies in animal experimental models by Holmgren, O'Reilly and Folkman[6] and O'Reilly et al[7] have identified circulating angiogenic inhibitors which are secreted from the primary tumour and suppress the growth of metastases. CTR is thought to explain the mechanism of the rapid progression of remote metastases after primary removal of the tumour. Although many physicians believe that CTR is present in certain types of cancer, research on CTR has been exclusively limited to animal models and no clinical studies have been carried out.

We have found CTR to be present in osteosarcoma using animal experimental models.[8] The removal of the primary osteosarcoma resulted in the progression of pulmonary metastases by angiogenic activation, suggesting a linkage between post-operative angiogenic activation and relapse of the disease. In this study, we have studied clinically the effects of removal of the primary tumour on the development of pulmonary metastases, the systemic angiogenesis-inducing ability, and the serum levels of several modulators of angiogenesis.

Patients and Methods

Patients and sample processing. Our study was approved under the institutional guidelines for the use of human subjects in research. Patients were considered to be eligible if they met the following criteria: a) the presence of biopsy-proven, primary high-grade osteosarcoma; b) tumour located in a limb; c) no evidence of metastases at diagnosis by bone scanning and CT of the lung; d) participation in the NECO-95J multi-institutional treatment protocol consisting of pre-operative chemotherapy, wide excision of the tumour, and post-operative chemotherapy,[9] and e) no associated diseases or conditions requiring medication of antiangiogenic reagents. In the NECO-95J protocol, high-dose methotrex-

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Table I. Details and clinical course of the ten patients with osteosarcoma

Case	Age (yrs)	Gender	Pulmonary metastases	Follow-up time (mths)	Clinical results*
1	10	F	No	41	NED*
2	10	M	No	42	NED
3	9	M	No	36	NED
4	14	M	No	35	NED
5	52	M	No	35	NED
6	18	M	10 mths after surgery	25	DOD
7	11	M	2 mths after surgery	28	DOD
8	14	M	5 mths after surgery	11	DOD
9	50	M	2 mths after surgery	16	DOD
10	16	M	11 mths after surgery	20	DOD

* NED, no evidence of the disease; DOD, died of the disease

ate, doxorubicine, cisplatin and ifosfamide are used in combination. The endpoint of this protocol is its completion or the development of distant metastases.

Between April 1999 and December 2001, 13 patients presented with osteosarcoma. Of these, one refused to receive chemotherapy and two had metastases at the time of diagnosis. These were excluded and the remaining ten patients were enrolled in the study (Table I). There were nine males and one female ranging in age from 9 to 52 years. Five of the tumours were in the femur and five in the tibia.

Peripheral venous blood samples were taken before biopsy (pre-operative serum) and two weeks after the resection of the tumour (post-operative serum). The serum was separated and stored at -80˚C and thawing was avoided until the assays were performed. The development of pulmonary metastases was evaluated monthly by plain radiography and every three months by CT. All patients reached the endpoint of the NECO-95J protocol and were followed up for a mean of 28.9 months (11 to 42). They were divided into two groups. The first consisted of those who did not develop metastases (no-recurrence group, cases 1 to 5, Table I) and the second those who developed pulmonary metastases within one year of primary excision (recurrence group, cases 6 to 10, Table I).

Matrigel plug assay. Systemic angiogenesis-inducing ability of the patients was measured by the Matrigel plug assay using the serum of the patients in an experimental animal system.

Six-week-old female BALB/c nu/nu mice (CLEA Japan Inc, Tokyo, Japan) were injected subcutaneously with 0.5 ml of Matrigel (Becton Dickinson, Bedford, Massachusetts) containing 50 µl of serum from the patients. The Matrigel pellets were harvested one week after the inoculation and reliquefied by incubation in 300 µl of phosphate-buffered saline at 4˚C overnight. Neovascularisation was determined quantitatively by measuring the haemoglobin content of the liquefied pellets (Drabkin's method; Sigma Chemical Co, St Louis, Missouri).[10,11] In experiments to evaluate the effects of endostatin, 50 ng of recombinant human endostatin[12] (Calbiochem, San Diego, California) were added to the Matrigel plug containing post-operative serum from the patients in the recurrence group. Each experiment was performed in triplicate, giving 15 samples for each group.

Enzyme-linked immunosorbant assay. The concentration of several angiogenic modulators (vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placenta growth factor (PlGF), endostatin and transforming growth factor-β1 (TGF-β1) in the sera from the patients was measured using an enzyme-linked immunosorbant assay kit as previously described[13] (VEGF, IBL, Fujioka, Japan; endostatin, Cytimmune Science Inc, College Park, Maryland; bFGF, PIGF and TGF-β1, R&D Systems, Minneapolis, Minnesota). VEGF and endostatin were measured in the pre- and post-operative serum and bFGF, PIGF and TGF-β1 in the pre-operative serum.

Immunohistochemical staining. The expression of CD34 in the biopsy specimens was determined using the avidin-biotin complex method as described before.[14] The primary antibody for CD34 (Nichirei, Tokyo, Japan) was applied at a dilution of 1:100. The number of CD34-positive vessels was counted in four randomly selected areas of a 1 mm^2 field and the mean number was referred to as microvessel density.

Evaluation and statistical analysis. Angiogenesis-inducing ability was analysed with respect to the correlation with the microvessel density of the biopsy specimens, the effect of removal of the primary tumour and the effect of endostatin. For analysis of the correlation between angiogenesis-inducing ability and the microvessel density, the concentration of haemoglobin in the pre-operative serum was measured by the Matrigel plug assay. Microvessels were counted after immunohistochemical staining of the biopsy specimen. The correlation was determined using the Spearman rank correlation test. The effect of removal of the primary tumour was determined by calculation of the post-operative increase in the systemic angiogenesis-inducing ability (subtraction of the concentration of haemoglobin in the pre-operative serum from that in the post-operative serum). The values of the post-operative increase in the systemic angiogenesis-inducing ability were compared between the no-recurrence and the recurrence group and analysed statistically using the unpaired *t*-test. In this analysis, each group contained 15 values (triplicate experiments for five patients). The effect of endostatin was similarly evaluated using the results of the Matrigel plug assay in the presence of human endostatin instead of the concentration of haemoglobin in the post-operative serum without endostatin.

The levels of angiogenic modulators in the pre-operative sera were compared between the no-recurrence and the recurrence groups, and the differences were analysed using the unpaired *t*-test. The levels of VEGF and endostatin were assessed post-operatively and the effect of removal of the primary tumour was determined by calculation of the post-operative changes (division of the differences between the post-operative and pre-operative serum levels by pre-operative serum level). In order to reduce the effect of VEGF pro-

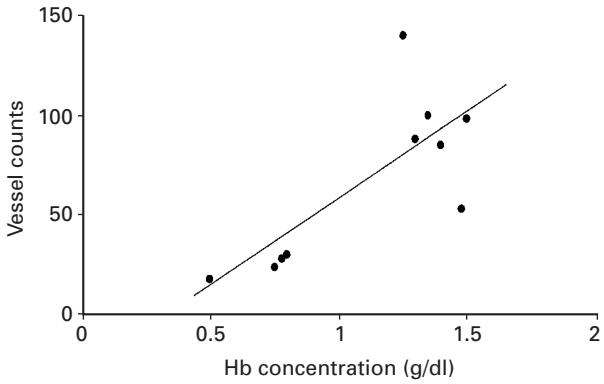

Fig. 1

Correlation between serum angiogenesis-inducing ability and vasculature of the primary tumour (Spearman rank correlation test).

duction by platelets,[15,16] the VEGF levels were also divided by the number of platelets (VEGF/Plt). The values of post-operative change were compared between the no-recurrence and the recurrence groups and analysed statistically using the unpaired *t*-test.

The response to pre-operative chemotherapy was evaluated using surgical specimens of the primary tumour and was divided into good and poor according to the criteria of the Japanese Orthopaedic Association.[17] The association between the chemosensitivity and the development of pulmonary metastases was determined by Fisher's probability test. In all analyses, statistical significance was defined as $p < 0.05$.

Results

We first evaluated the correlation between the angiogenesis-inducing ability of the pre-operative serum and microvessel density in the primary tumour. As shown in Figure 1, there was a significant correlation ($r = 0.672$, $p = 0.016$) suggesting that the angiogenesis-inducing ability of the serum reflects the neovascularisation status of the tumour.

We then evaluated the effect of the primary tumour on the regulation of systemic angiogenesis-inducing ability. As shown in Figure 2, the latter increased after removal of the tumour in all ten patients with osteosarcoma, and the post-operative increase was significantly higher in the recurrence group (0.84 ± 0.39) (mean ± SD), than in the no-recurrence group (0.06 ± 0.03). This increase disappeared after addition of recombinant human endostatin in the post-operative serum of the recurrence group (0.1 ± 0.08, $p = 0.06$). With respect to the response to pre-operative chemotherapy, two patients were defined as good responders in the recurrence group and three patients in the no-recurrence group. There was no statistical difference.

It is well known that the process of angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors.[18] The post-operative levels of VEGF, VEGF/Plt and endostatin were found to be significantly higher in the recurrence group than the no-recurrence group. By contrast, there were no such differences in the serum levels of bFGF, P1GF and TGF-β1 (Table II). Subsequently, we evaluated the effect of removal of the primary tumour on the regulation of VEGF and endostatin. In all patients, the serum levels of VEGF and endostatin decreased after removal of the tumour (Fig. 3). The post-operative change in the serum levels of VEGF and VEGF/Plt was higher in

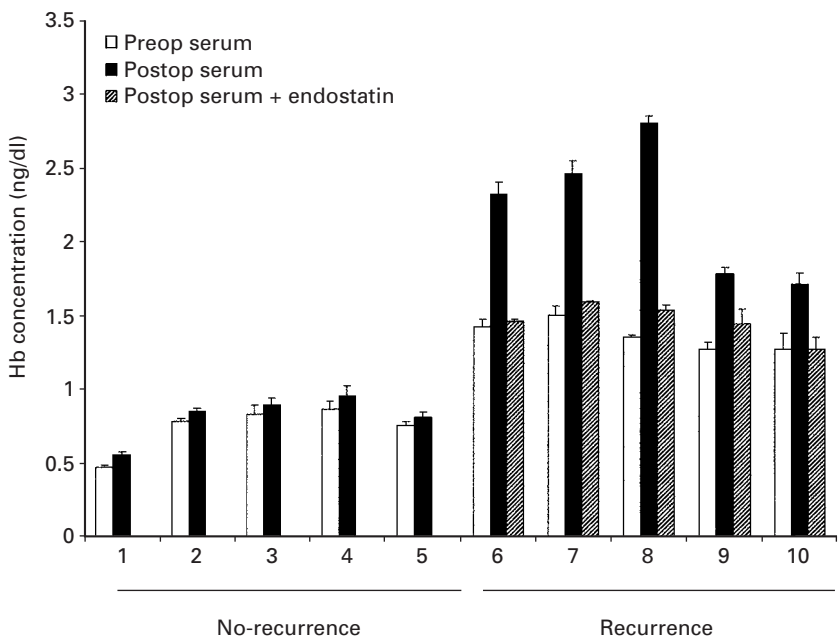

Fig. 2

The effects of removal of the primary tumour on systemic angiogenesis-inducing ability. The results of triplicate experiments were shown by the mean ± SD in each patient.

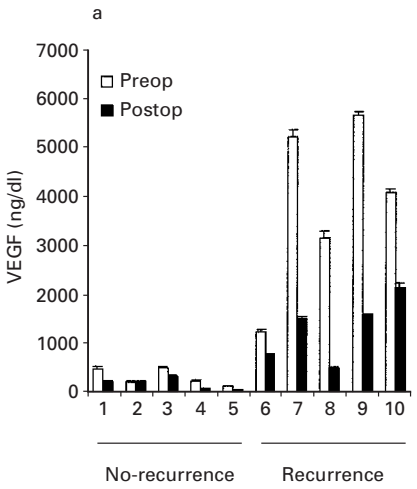

Fig. 3a

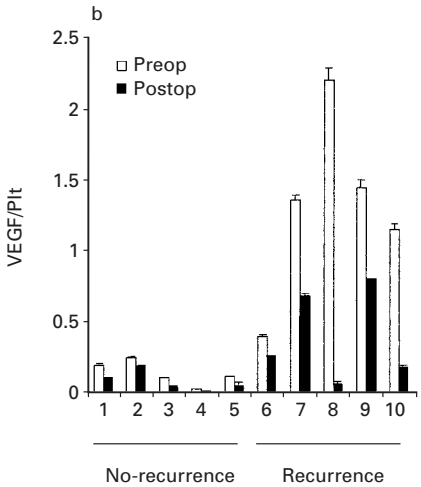

Fig. 3b

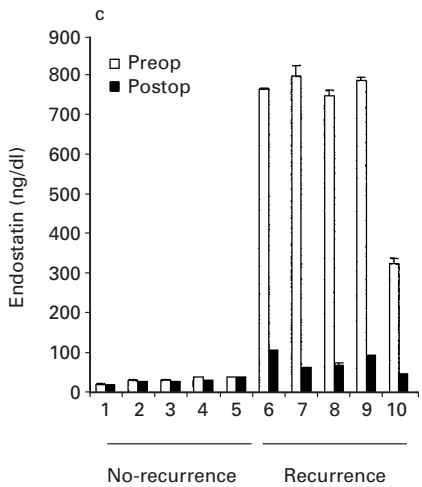

Fig. 3c

Effects of removal of the primary tumour for serum on the mean serum (± SD) levels of a) VEGF, b) VEGF/Plt and c) endostatin.

Table II. Mean (± SD) pre-operative serum level of angiogenic modulators

Angiogenic modulators	No-recurrence group (n = 5)	Recurrence group (n = 5)	p value
VEGF (pg/ml)	336.8 ± 164.9	3890.0 ± 1766.4	0.001
VEGF/Plt (pg/10^6Plt)	0.13 ± 0.09	1.31 ± 0.65	0.002
P1GF (pg/ml)	89.9 ± 132.3	102.1 ± 115.2	0.440
bFGF (pg/ml)	171.0 ± 133.2	165.8 ± 112.4	0.473
Endostatin (ng/ml)	34.0 ± 6.98	685.1 ± 203.5	0.000047
TGF-β1 (pg/ml)	81.3 ± 90.7	38.6 ± 13.3	0.164

Table III. Reduction ratio* of VEGF, VEGF/Plt and endostatin

	No-recurrence group (n = 5 x 3)	Recurrence group (n = 5 x 3)	p value
VEGF	41.3 ± 25.6	61.9 ± 19.9	0.097
VEGF/Plt	39.4 ± 20.5	57.6 ± 19.5	0.094
Endostatin	11.4 ± 2.92	86.5 ± 3.14	0.000001

* division of the differences between pre-operative and post-operative serum levels by pre-operative serum level

the recurrence than the no-recurrence group, but was not statistically significant (Table III). By contrast, the post-operative change in the serum levels of endostatin was significantly higher in the recurrence group than in the no-recurrence group (Table III).

Discussion

We have determined the association between the post-operative increase of angiogenic activity and the progression of pulmonary metastases in patients with osteosarcoma. This is the first supportive clinical evidence of the presence of CTR.

Osteosarcoma is a high-grade malignant tumour with biological features which are advantageous for the clinical analysis of CTR. These include the existence of micrometastases on initial presentation at a uniform metastatic stage,[4] and the relapse of micrometastases during the first year of treatment, especially after excision of the primary tumour. Nevertheless, by using a Matrigel plug assay, a link between recurrence and systemic angiogenesis may also be defined in a variety of cancers other than osteosarcoma, which will further verify the presence of CTR in patients with cancer in analyses on a larger scale.

With regard to the mechanism underlying CTR, Holmgren et al[6] have proposed a critical role for angiogenic inhibitors such as endostatin in the regulation of micrometastatic dormancy. In support of this, we have found that removal of the primary tumour decreased the serum level of endostatin thereby presumably withdrawing systemic angiogenic inhibition. Moreover, recovery of serum endostatin in the post-operative serum decreased the angiogenesis-inducing ability to pre-operative levels in the recurrence group. It should, however, be noted that removal of the primary tumour also decreased the serum level of VEGF, an angiogenic promoter, indicating a paradoxical role of removal of the tumour. This may be partly explained by the shorter half-life of VEGF relative to endostatin.[19] Furthermore, the rate of decrease was more obvious for endostatin than for VEGF in comparison between the recurrence and no-recurrence groups.

Angiogenesis is essential for the establishment of remote metastases, leading to the concept of angiosuppression as a new therapeutic strategy for the treatment of cancer. Several clinical trials have been started using antiangiogenic reagents.[20-27] Regarding osteosarcoma, we have previously shown that patients with a VEGF-positive tumour developed

pulmonary metastases more often than did those with a VEGF-negative tumour.[14] Furthermore, the serum level of VEGF in patients who had pulmonary metastases within one year after removal of the primary tumour was significantly higher than that of patients who did not.[13] These findings suggest that the establishment of pulmonary metastasis in osteosarcoma is dependent on, at least in part, angiogenesis and by using antiangiogenic methods, the progression of pulmonary metastases in patients with osteosarcoma may be prevented. Therefore, our findings suggest that patients with osteosarcoma in whom systemic angiogenesis is elevated post-operatively will be good candidates for antiangiogenic therapy. This may allow patients with osteosarcoma to coexist with dormant metastases and will improve their outcome. These findings will require confirmation by large-scale analysis but we suggest that they present a new challenge to the treatment of osteosarcoma.

Because of the small sample size of our study several questions remain unanswered. These include the effect of heterogeneity in the gender and age of the patients and post-operative chemotherapy on the regulation of CTR and the effect of the extent of the post-operative increase in angiogenesis-inducing ability on the timing of post-operative recurrence. Further studies on a larger scale will answer these important questions.

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