Journal of Surgical Oncology 1998;68:169–172

Assessment of Chemosensitivity in Patients With Osteogenic Sarcoma Using the Doxorubicin Binding Assay

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Background and Objectives: Assessment of chemosensitivity in patients with osteosarcoma may help identify those with resistance to chemotherapy. In this study, we investigated the clinical value of the doxorubicin binding assay in its ability to identify patients with drug resistance.
Methods: We tested tumor tissue samples obtained at biopsy of 24 patients with high-grade osteosarcoma aged 9–61 years (mean 19.2) for sensitivity to doxorubicin, using the doxorubicin binding assay. Tumor excision was performed in these patients after neoadjuvant chemotherapy. Chemotherapy response was judged on the basis of tumor necrosis achieved and was compared with doxorubicin sensitivity in each of these patients.
Results: Doxorubicin sensitivity was good in 15 and poor in 9 of 24 patients studied. In patients with good sensitivity (n = 15), 9 (60%) exhibited a good response to chemotherapy while response was poor in 6. In patients with poor sensitivity (n = 9), response to chemotherapy was poor in all 9 (100%) patients and 7 (77.8%) of these patients developed metastatic disease within a mean period of 5.2 months, resulting in two deaths. The results were statistically significant at $P = 0.0193$.
Conclusions: Doxorubicin binding assay may be useful in identifying patients with inherent resistance to chemotherapy. As the outcome of patients showing resistance to doxorubicin is poor, innovative strategies may need to be developed for this group of patients.
J. Surg. Oncol. 1998;68:169–172.

KEY WORDS: **bone; sarcoma; drug resistance**

INTRODUCTION

Osteosarcoma is the commonest primary malignant tumor of bone. The survival of patients with this disease has improved over the past decade, primarily as a result of effective chemotherapy and improved surgical management [1]. Appropriate preoperative chemotherapy allows the surgeon to perform limb-sparing procedures in these patients; the drug-induced tumor necrosis enhances the effect of local control obtained through surgery [2,3]. The response to chemotherapy, however, can only be judged when the tumor is excised, and the surgical specimen is histologically examined to determine the extent of necrosis [4]. This may be too late in the case of patients displaying drug resistance or poor response to chemotherapy, as valuable time may be lost before remedial measures can be instituted. Preliminary assessment of chemosensitivity in osteosarcoma patients may help identify patients with inherent multidrug resistance

Grant sponsor: Chinese University of Hong Kong; Grant number: CUHK MR 94/010.

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Accepted 16 March 1998

TABLE I. Doxorubicin Binding Assay Study: Patient Data*

Patient no.	Sex/ Age	Tumor site	Sensitivity	Tumor necrosis	Status	Follow-up (mo)
1	F/14	Femur	Resistant	Poor	DOD	5
2	F/26	Femur	Resistant	Poor	AWD	12
3	M/10	Tibia	Resistant	Poor	DOD	5
4	M/45	Femur	Resistant	Poor	AWD	9
5	M/21	Pelvis	Resistant	Poor	DOD	3
6	F/9	Femur	Resistant	Poor	NED	8
7	F/61	Femur	Resistant	Poor	AWD	7
8	F/9	Femur	Resistant	Poor	NED	4
9	F/47	Pelvis	Resistant	Poor	AWD	9
10	M/24	Pelvis	Sensitive	Poor	DOD	9
11	M/10	Femur	Sensitive	Poor	NED	29
12	F/22	Tibia	Sensitive	Poor	DOD	15
13	M/51	Femur	Sensitive	Poor	NED	7
14	M/17	Tibia	Sensitive	Poor	NED	7
15	M/12	Femur	Sensitive	Poor	NED	6
16	F/10	Femur	Sensitive	Good	NED	27
17	F/12	Femur	Sensitive	Good	NED	24
18	M/12	Tibia	Sensitive	Good	AWD	20
19	F/12	Femur	Sensitive	Good	NED	17
20	F/11	Tibia	Sensitive	Good	NED	9
21	F/9	Tibia	Sensitive	Good	NED	4
22	M/17	Femur	Sensitive	Good	NED	12
23	M/13	Femur	Sensitive	Good	NED	8
24	F/12	Femur	Sensitive	Good	NED	8

*NED, no evidence of disease; AWD, alive with disease; DOD, died of disease.

(MDR) to chemotherapy so that alternative treatment options could be considered.

MDR is characterized by a loss of sensitivity to a variety of structurally unrelated drugs, including doxorubicin. The MDR phenomenon may present at clinical onset of the tumor or may develop during the course of the disease [5]. It is characterized by the overexpression of P-glycoprotein, a membrane protein associated with intracellular drug levels [6]. Doxorubicin is very effective in high-grade osteogenic sarcoma and should be an essential part of any multimodal therapy for this tumor [7]. The doxorubicin binding assay [8] was developed as a simple chemosensitivity assay based on different patterns of intracellular doxorubicin distribution. We have used this assay to determine its clinical value in screening patients who are unlikely to respond to chemotherapy.

MATERIALS AND METHODS

We tested tumor tissue samples (obtained at diagnostic biopsy) from 24 patients with high-grade osteosarcoma aged 9–61 years (mean 19.2) for sensitivity to doxorubicin (Table I). After a complete diagnostic workup that comprised whole-body scintigraphy, thoracic computed tomography (CT), and magnetic resonance imaging (MRI) of the affected limb, followed by an open biopsy, the disease was confirmed and staged according to the classification proposed by Enneking et al. [9]. All patients presented with stage IIB disease, and the tumor was located in the pelvis in three patients, the tibia in six patients, and the femur in 16. Patients were followed up in a joint oncology-orthopedic clinic at the Prince of Wales Hospital for periods ranging from 5 months to 29 months (mean 11 months). Tumor tissue samples from the patients were obtained at the time of the surgical biopsy. Doxorubicin binding assay was performed on these tissue samples, using a technique previously described by Baldini et al. [8].

TREATMENT

All patients were given neoadjuvant chemotherapy using a standard combination of high-dose methotrexate, cis-platinum, and doxorubicin after biopsy and staging studies were completed. After completion of chemotherapy, the tumor was resected and histologically examined for tumor necrosis. The chemotherapy response was graded I–IV depending on the extent of tumor necrosis [4]. Since tumor necrosis correlates well with long-term prognosis and survival [2–4], we compared the sensitivity of these patients as assessed by the doxorubicin binding assay method with the percentage of tumor necrosis achieved.

Doxorubicin Binding Assay

Doxorubicin is a natural fluorochrome; intracellular drug accumulation may be monitored by direct microscopic observation. Different patterns of drug distribution have been observed in living sensitive and resistant cells. This is the basis of the doxorubicin binding assay.

The representative tumor sample was selected and placed immediately in Isocove's modified Dulbecco's medium (Sigma I-7633, Sigma Chemical Co., St. Louis, MO). After a brief mechanical dissociation, the sample was treated with 0.1% collagenase II (Sigma C-6885) in phosphate-buffered saline (PBS) at 37°C, until an adequate cell suspension was obtained. Tissue debris was removed after filtration through a 100-μm Whatman filter. After the addition of further culture medium to dilute the collagenase, the suspension was centrifuged at 1,000 rpm for 5 min. The supernatant fluid was taken away and an appropriate measured volume of culture medium added to resuspend the cells. The cells per unit volume were determined using a counting square. Cell viability was then calculated using the trypan blue exclusion method. A total of 200,000 cells from the cell suspension were resuspended in 1 ml of culture medium.

Doxorubicin (10 mg) was dissolved in 5 ml of double-distilled water to obtain the desired concentration of 10 μg/ml; 5 ml of this solution was added to 1 ml of the suspension of cells obtained and incubated at 37°C for 30 min under continuous motion. A total of 2 ml of fluoroscein diacetate solution (Sigma F-7378, 0.5 mg/ml, pH 7.3) was added with continuous stirring at 60°C and un-

der dark conditions for 10–15 min. Next, 1 ml PBS was added and the fluid centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in 50 ml PBS and 10 ml placed on a slide for fluorescence microscopy.

Fluorescence microscopy for the doxorubicin binding assay [10] was performed as follows. Living cells were identified using fluorescein diacetate fluorescence. Under blue excitation, the living cells show green fluorescence, whereas the nonviable cells do not. The red fluorescence of doxorubicin was then recorded under red excitation conditions.

The fluorescence was simultaneously read by a research student and two scientific officers with many years' experience in fluorescence microscopy. The study was blinded in such a manner that the persons involved with the doxorubicin binding assay had no access to patient's clinical details, including stage and grade of the tumor, or the percentage of tumor necrosis observed following chemotherapy. The results of the doxorubicin binding assay were also witheld from the clinicians and the pathologist in charge of measuring necrosis of the resected tumor, until a final analysis of the study was performed.

Two types of patterns were observed as follows: type A, strong nuclear fluorescence with weak fluorescence in the cytoplasm; and type B, weak diffuse fluorescence in the cytoplasm without any distinctive intracellular binding. Sensitive cells showed the type A binding pattern, while resistant cells displayed the type B binding pattern. The percentage of sensitive cells was calculated counting 300 cells for each sample. The tumor was considered chemosensitive if more than 80% of the cells showed nuclear fluorescence or a type A binding pattern.

Statistical Analysis

Statistical analysis was done using the *t*-test on a proprietary statistics program (Statisticia version 4.1, Statsoft, Tulsa, OK, USA, 1994).

RESULTS

ABA Positive Group

Adriamycin Binding Assay (ABA) was positive in 15 of 24 patients. Nine of these 15 patients (60%) showed grade III–IV necrosis, indicating a good response to chemotherapy, while the remaining 6/15, (40%) had a poor response with only grade I–II necrosis. Of the good responders, only one patient has developed metastatic disease, while three of the six poor responders have developed metastatic disease (Table II).

Doxorubicin Binding Assay-Negative Group

Doxorubicin binding assay sensitivity was poor in 9 of the 24 patients. All these patients exhibited poor response to chemotherapy. Seven (77.8%) of these nine patients developed metastatic disease within a mean period of 5.2 months after surgery (range 3–8 months). Disease progression was rapid in two patients with metastasis, both of whom died within 5 months after surgery. Only two patients remained disease free at 4 and 8 months follow-up. Doxorubicin binding assay sensitivity was found to be significant at $P = 0.0193$.

TABLE II. Doxorubicin Binding Assay: Sensitivity, Tumor Necrosis, and Number of Patients Who Developed Metastases in Assay-Sensitive vs. Assay-Resistant Patients*

Doxorubicin binding assay	Tumor necrosis	
	Good	Poor
Sensitive (n = 15)	9	6
metastases	1/9 (11.1%)	3/6 (50%)
Resistant (n = 9)	0	9
metastases	—	7/9 (77.8%)

*Doxorubicin binding assay sensitivity statistically significant at $P = 0.0193$.

DISCUSSION

Ineffectiveness of tumor-directed chemotherapy is often caused by resistance of malignant cells to a wide range of cytotoxic drugs. The main characteristic of these drug-resistant cells is an energy-dependent outward transport of drugs by a nuclear membrane protein known as P-glycoprotein [11,12]. Another key feature is that this MDR is potentially reversible by agents such as verapamil [13] and calmodulin inhibitors [14]. However, the application of such therapy is only in the trial phase at the moment [15]. Thus, reliable estimation of drug resistance before the onset of chemotherapy may allow the clinician to change the strategy toward patients with drug resistance.

The doxorubicin binding assay provides valuable information regarding the sensitivity of patients towards neoadjuvant chemotherapy. Its interpretation, however, needs further explanation. Our data indicate that patients with resistance to doxorubicin have a poor outcome in terms of chemonecrosis and metastatic disease. Such patients may well benefit by the application of drug-reversal strategies, which at the moment are only in the trial stage. In the absence of such reversal agents, chemotherapy may only delay the process of surgical excision, thereby allowing resistant cell clones to proliferate. It may therefore be prudent to abandon neoadjuvant chemotherapy in this group of patients with resistance to doxorubicin and embark upon immediate surgical excision instead.

It is well known that patients with initial high chemosensitivity often acquire resistance to drugs during the course of treatment [16]. Chemotherapy eliminates the sensitive cells while allowing resistant cells to multiply and eventually metastasize. Thus, sensitivity to doxorubicin does not necessarily ensure a predictable response

to chemotherapy. Despite good sensitivity, 40% (6/15) of our patients developed resistance and showed a poor response to chemotherapy, as judged by the tumor necrosis achieved. By contrast, doxorubicin resistance almost certainly means an unfavorable outcome. All 9 patients with doxorubicin resistance had poor response in terms of chemonecrosis, 7 (77.8%) of whom went on to develop metastatic disease. In this challenging group of patients, new approaches and aggressive treatment protocols may need to be developed in the future.

CONCLUSIONS

The doxorubicin binding assay is a useful test to screen patients in whom the response to chemotherapy is likely to be poor. Only 60% of patients with good sensitivity (doxorubicin binding assay positive) may respond well to chemotherapy, while 40% may eventually develop resistant disease. In patients with poor sensitivity (doxorubicin binding assay negative), response to chemotherapy is almost certain to be poor and, in this challenging group of patients, innovative strategies to overcome or reverse drug resistance may need to be applied.

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