In Vitro Chemosensitivity Assay for Soft Tissue Sarcoma Using Tumor Xenografts

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ABSTRACT

The authors have investigated the clinical usefulness of an in vitro chemosensitivity assay for soft tissue sarcoma using tumor xenografts. Biopsy or surgical specimens from 47 patients with soft tissue sarcoma were transplanted into nude mice. In vitro chemosensitivity of each patient was determined using these early-generation xenografts by means of in vitro scintillation assay. Thirty-three of 47 clinical samples (70.2 %) obtained from patients were successfully transplanted into nude mice. Of these 33 xenografts, 25 met the criterion for in vitro growth. Overall, 25 of 47 (53 %) samples gave a successful assay. Twenty-four of the 47 patients died of metastatic disease at a median of 26.3 months (range, 2 to 86 months). Five patients were excluded from this study: one patient died of chemotherapy side effects, one died of lung cancer and the other three were dropped out. The average follow-up period of 18 surviving patients was 85 months (range, 60 to 116 months). A significant difference in the five-year survival rate was noted between the patients with a successful assay (36.6 %) and those with a failed assay (56.1 %). Retrospectively, the in vitro results were compared with the clinical responses of 16 patients who received systemic chemotherapy and operation. The true positive rate, true negative rate, and predictive accuracy were 33 %, 100 %, and 75 %, respectively. The authors suggest that this in vitro chemosensitivity assay system provides a valid tool for prognosis and facilitates the exclusion of ineffective drugs for treating cases of soft tissue

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sarcoma. As a result, a more efficient therapy for this disease may be obtained.

Key words : Chemosensitivity, Soft tissue sarcoma, In vitro, Xenograft, Scintillation assay

INTRODUCTION

Soft tissue sarcomas are rare and complex tumors of mesenchymal origin. Although most patients present with apparently localized disease, which allows good local control, about 50 % die from subsequent metastases[1].

The use of chemotherapy in soft tissue sarcoma is a matter of intense debate despite its proven degrees of efficiency in treating osteosarcomas and Ewing sarcomas[2]. For the time being, only two drugs - doxorubicin[3] and ifosfamide[4] have consistently shown successful single agent activity in more than 20% of patients who did not receive pretreatment. Recently, meta-analysis has provided evidence that adjuvant doxorubicin-based chemotherapy significantly improves the time to local and distant recurrence and overall recurrence-free survival[5]. However, there was little evidence that certain types of patients benefited more or less from adjuvant chemotherapy. It would be of great interest to predict the chemosensitivity of individual tumors using a laboratory test.

Although various in vitro chemosensitivity assays for many kinds of cancers have been reported[6], there have been very few published reports of their use for adult soft tissue sarcomas[7]. The major limitation of in vitro chemosensitivity assay of sarcomas can be described as the difficulty in maintaining primary cultures of sarcoma cells in vitro, resulting in low assay-success rates.

The authors previously reported an in vitro chemosensitivity assay for osteosarcoma[8,9]. To overcome the difficulty in maintaining primary osteosarcoma cells in culture, biopsy or surgical specimens were transplanted directly into athymic nude mice and early-generation xenografts were used for an in vitro scintillation assay. In this study, the authors applied this assay system to soft tissue sarcoma and investigated its reliability and clinical availability. A successful assay proved to be a poor prognostic indicator for the individual patients.

MATERIALS AND METHODS

Patients and Clinical Samples

Tumor specimens were obtained from 47 patients with soft tissue sarcoma treated at the Department of Orthopaedic Surgery, Sapporo Medical University and the Department of Orthopaedic Surgery, National Sapporo

Hospital between January 1,1989 and July 31,1991. Pathological diagnosis and primary site of the tumors are shown in Table 1. During the time-period of

Table 1 Pathological diagnosis and primary site of the tumor for which chemosensitivity was tested

	No. of patients
Pathological diagnosis	
MFH	14
Liposarcoma	10
Neurosarcoma	6
Rhabdomyosarcoma	4
Leiomyosarcoma	4
Synovial sarcoma	2
Epithelioid sarcoma	2
Extraskeletal osteosarcoma	2
Others	3
Primarysite	
Extremity	38
Trunk	9

the study, we performed an operation on 60 patients who had a soft tissue sarcoma involving the extremities and trunks. The only inclusion criterion for the study was an appropriate amount of the tumor specimen obtained from the operation. Thirteen specimens were taken from biopsy materials after obtaining the appropriate informed consent and thirty-four were from definitive surgery. Of the 34 surgical specimens, 33 were from primary lesions and one was from a metastatic lesion. In 16 patients, tumor specimens were obtained after patients had received at least one course of chemotherapy. Soft tissue sarcoma tissues were transferred directly to in vitro cultures and, at the same time, were transplanted into athymic nude mice as described previously[8].

Twenty-four of the 47 patients died of metastatic disease at a median of 26.3 months (range, 2 to 86 months). The average follow-up period of 18 surviving patients was 85 months (range, 60 to 116 months). The overall five-year rate of survival was 47 per cent.

Tissue Collection and Preparation

About 2 × 2 × 2cm of soft tissue sarcoma tissue was minced to a fine consistency. 0.5-ml sample of the minced tissue was injected subcutaneously into the backs of the BALB/C (nu/nu) athymic nude mice. From one to three animals were used per tumor, depending on the specimen size. The maximum time from surgical resection to injection into the nude mice was 2 hours. The same technique was used for successive serial transplants. Only early generation sarcoma xenografts were used for in vitro chemosensitivity tests. Sections of solid tumor samples from the patients or nude mice were disaggregated by fine mincing followed by exposure to enzymes. The enzyme solution consisted of a mixture of 0.2% collagenase and 0.1% dispase (Sigma, St. Louis, MO). Following incubation in the enzyme solution at 37℃ for 30 minutes, the cell suspension was filtered through gauze and a 200 mesh stainless steel screen. The cells were centrifuged at 1200 rpm for 10 minutes and resuspended in 10% fetal calf serum (ICN, Costa Mesa, CA) containing DMEM (Nissui, Tokyo). The number of cells was adjusted to 3×10^5/ml.

Drugs

All of the drugs investigated were obtained from commercial sources. Abbreviations for the 14 antineoplastic reagents used are shown in Table 2. Drugs were freshly prepared every 4 months and stored at -70℃. Table 2

Table 2 Drug concentration at which chemosensitivity was tested

Drugs	Abbreviation	Concentration (μg/ml)
Actinomycin-D	DACT	0.005
Amrubicin	SM5887	0.04
Bleomycin	BLM	0.1
Carboplatin	CBP	0.2
Cis-platin	CDDP	0.2
Cyclophosphamide	CPM	3.0
Doxorubicin	ADR	0.04
Epirubicin	EPI	0.04
Etoposide	VP16	3.0
Ifosfamide	IFO	3.0
Methotrexate	MTX	20.0
Mitomycin C	MMC	0.1
Piarubicin	THP	0.04
Vincristin	VCR	0.01

shows the drug concentration at which chemosensitivity was evaluated. The single concentration of each drug selected corresponded to approximately 1/10 of the peak plasma concentration for each drug in humans when high dose methotrexate (MTX) is used clinically. For cyclophosphamide, 4-hydroxy cyclophosphamide was used as an active form. Peak pharmacologically achievable concentrations of the drugs were determined according to the theory of Von Hoff et al[6].

In vitro Chemosensitivity test

The assay system used in this study has been described previously[8,9]. In brief, cells were plated into 96 well microplates at a density of 3×10^4 cells per well. Plated assays were incubated at 37℃ in the presence of 5% CO_2. After 24 hours of incubation, 4 serial dilutions of the antineoplastic drugs were added to the well. Control wells were plated without the addition of any cytotoxic reagents. After 24 hours of exposure to the chemotherapeutic agents, the supernatant was discarded and fresh medium was added to each well. The plates were then incubated for another 48 hours. Then 0.5 μCi of [^{3}H] - deoxyuridine (^{3}H-UdR) (NEN, Wilmington, DE) was added to each well. After 4 hours of incubation, the cells were treated with trypsin (Sigma, St. Louis, MO) and harvested with a cell harvester. Counts per minutes (cpm) for drug treated and control wells were determined with a Beckmann liquid scintillation counter. All experiments were set up in quadruplicate and the mean value was used to calculate radioactivity incorporation. Percent deoxyuridine incorporation was calculated by dividing the cpm of the treated wells by the cpm of the control wells.

The criterion of a valid growth assay is the incorporation of more than 300 cpm of ^{3}H-UdR in the control wells. Sensitivity was assayed at the drug concentrations shown in Table 2. In vitro sensitivity was evaluated as effective when percent ^{3}H-UdR incorporation was $\leq$ 25%[8].

Clinical Chemotherapy

Chemotherapeutic drugs given to each assessable patient are shown in Table 3. Ten patients received preoperative chemotherapy. Thirteen patients received postoperative adjuvant chemotherapy. Among them, 7 patients received both pre and postoperative chemotherapy. Fourteen of 16 patients were treated with combination chemotherapy rather than single-drug therapy. During the time period of the study, high dose ifosfamide was not administered to the patients.

Table 3 In vitro and in vivo results of 16 patients who had received chemotherapy

Case	Diagnosis	Location of primary tumor	Surgical margin	Drugs given to patients[1]	pre/post[2]	Clinical response		Prognosis[5]	In vitro/ in vivo correlation[6]
						Response of the tumor[3]	Metastasis/ recurrence[4]		
1	Rhabd.	thigh	wide	SM5887	post	_	LM(4)	DOD(9)	R/R
2	Rhabd.	back	wide	DACT, VCR, CPM, ADR	pre/post	NC	LM(4)	DOD(8)	S/R
3	Rhabd.	forearm	wide	VCR, DACT, CPM, ADR, CDDP, VP-16	post	_	LM(30)	DOD(35)	S/S
4	MFH	back	marginal	ADR, CPM, CDDP	pre/post	PD	LM(13)	DOD(22)	R/R
5	MFH	thigh	wide	CPM, VCR, ADR, DTIC, BLM, CDDP	post	_	LM(7)	DOD(15)	R/R
6	Neurosa.	thigh	wide	ADR, CDDP	pre	NC	LM(86)	DOD(86)	S/R
7	Liposar.	thigh	wide	CDDP, CPM, VCR, ADR, DTIC	pre	NC	rec(35)	NED(110)	R/R
8	Epith.sa.	axilla	intra	CDDP, ADR, CPM, DACT, BLM, VCR	pre/post	PD	rec(4)	DOD(8)	S/R
9	Liposa.	thigh	wide	CPM, VCR, ADR, DTIC	pre	NC	rec(23)	NED(78)	R/R
10	Synov.sa.	thigh	wide	CPM, VCR, ADR, DTIC	post	_	LM(10)	NED(89)	R/R
11	Synov.sa.	foot	wide	ADR, MTX, CDDP, BLM, CPM, DACT	pre/post	NC	LM(35)	DOD(39)	S/R
12	Liposa.	thigh	marginal	CDDP, ADR	post	_	LM(12)	DOD(25)	R/R
13	Epith.sa.	elbow	wide	ADR, CPM, CDDP, VP16	pre/post	PR	no	CDF(60)	S/S
14	MFH	buttock	wide	SM5887	post	_	LM(2)	DOD(36)	R/R
15	Undeter.*	buttock	wide	ADR, CPM, VCR, DTIC	pre/post	NC	LM(1)	DOD(3)	R/R
16	MFH	buttock	wide	CPM, VCR, ADR, DTIC	pre/post	PD	rec(1)	DOD(9)	R/R

1) Drugs judged as sensitive by in vitro chemosensitivity assay are underlined.
2) Drugs were given preoperatively (pre) and/or postoperatively (post).
3) Clinical evaluation of the patient who received preoperative chemotherapy (see text).
PR: partial response, NC: no change, PD: progression of disease
4) Clinical evaluation of the patient who received only postoperative chemotherapy (see text).
LM: lung metastasis, rec: local recurrence.
Number in parentheses indicates time period (months) when a lung metastasis or a local recurrence developed after diagnosis.
5) CDF: continuous disease free, NED: no evidence of disease, DOD: dead of disease
Number in parentheses indicates follow up time period (months) after diagnosis.
6) S: sensitive, R: resistant

Clinical Assessment

To obtain clinical correlations, all patients were evaluated using following criteria.

1) Criteria for the patients who received preoperative chemotherapy.

Complete response (CR) was defined as complete disappearance of all

measurable disease for a period of at least 4 weeks; partial response (PR) was defined as ≥ 50% regression of measurable disease in the absence of new lesions; no change (NC) was defined as no change in measurable disease; progressive disease (PD) was defined as ≥ 25% progression of disease. Both CR and PR were accepted as responses.

2) Criteria for the patients who received only postoperative chemotherapy.

Response was defined as no local recurrence or distant metastasis within 24 months after diagnosis. If the patient received both preoperative and postoperative chemotherapy, clinical chemosensitivity was determined with regard to preoperative therapy.

Most of the patients were treated with drug combinations rather than with a single agent; therefore, it was difficult to make an accurate in vitro/in vivo correlation. The following definitions were used[8] : true positive (S/S), a clinical response was obtained by using 1 or more sensitive drugs in vitro; true negative (R/R), no clinical response was obtained by using only non-sensitive drugs in vitro; false positive (S/R), a clinical response was not obtained using at least one sensitive drug in vitro; and false negative (R/S), a positive clinical response was obtained by using only drugs which were not sensitive in vitro. The true positive rate, true negative rate, predicting accuracy, sensitivity and specificity were calculated as described by Clark and Von Hoff[6].

Statistical Methods

Survival curves were constructed with the Kaplan-Meier method and compared using the generalized Wilcoxon's test.

RESULTS

Technical Success Rate

Thirty-three of 47 clinical samples (70.2 %) obtained from patients were successfully transplanted into nude mice. Of these 33 xenografts, 25 met the criterion for in vitro growth. The main causes of assay failure in 14 xenografts were insufficient growth and low yield of viable tumor cells. Overall, 25 of 47 (53.1%) samples gave a technically successful assay.

Prognosis of Patients with Xenograft Establishment or Successful Assay

In order to study whether establishment of xenografts in nude mice is of prognostic significance, we divided the patients into groups depending on whether they had an established xenograft or not. There was a tendency toward poor prognosis in patients whose tumors successfully grafted in the

nude mice. However, no statistical significance was found (Fig 1A).

Next, we divided the patients into 2 groups, according to whether or not they had a successful assay. Five-year survival rates were 36.6 % for the group of patients whose tumors resulted in successful assay and 56.1 % for the group of patients whose tumors resulted in failed assay. The difference in survival rate was significant by generalized Wilcoxon's test ($p < 0.05$) (Fig 1B).

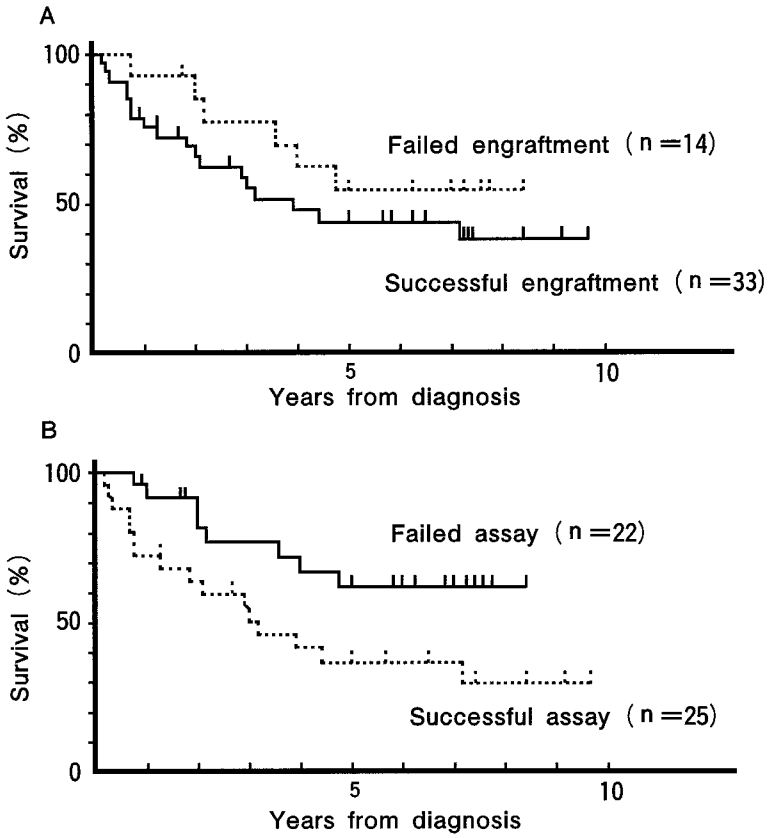

Fig. 1 Survival time of patients with soft-tissue sarcoma subdivided according to xenograft establishment (A) and successful assay (B).

In Vitro Results

The dose response curves of five representative patients are shown in Fig 2. Chemosensitivity was dose-dependent for all of the five patients. The tumor was considered responsive if $\geq$ 75 per cent inhibition of ^{3}H-UdR incorporation was achieved at the concentration of each drug shown in Table 2[8].

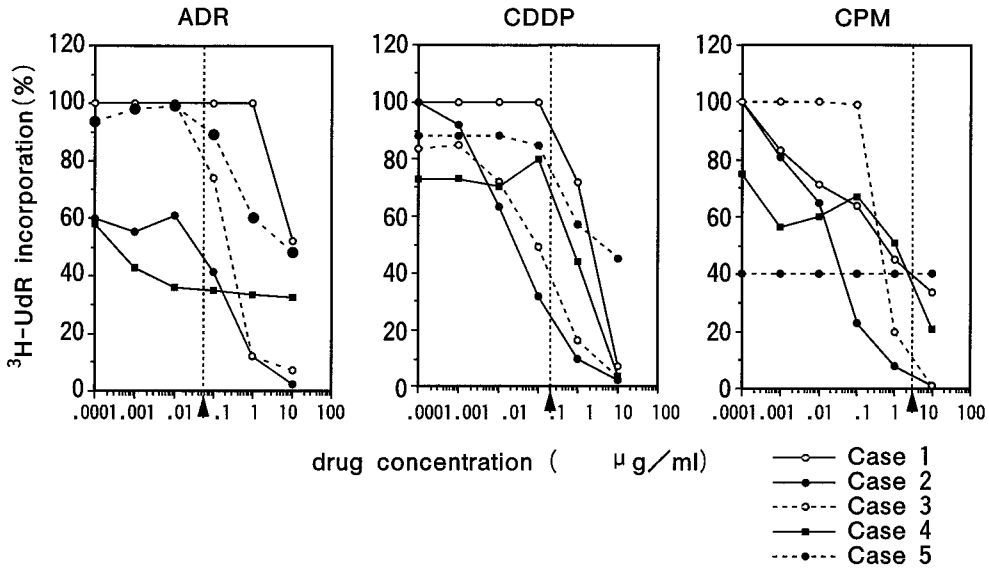

Fig. 2 Dose response curves of 5 representative patients with soft tissue sarcoma to doxorubicin (ADR), cis-platin (CDDP) and cyclophosphamide (CPM).
Chemosensitivity for each drug was evaluated at the concentration indicated with arrows on the horizontal axis. In vitro chemosensitivity was evaluated as effective when the percentage of ^{3}H-UdR incorporation was $\leq$ 25 %.

According to this criterion, Case 2 was responsive to CDDP and CPM. Case 3 was responsive to CPM. The other 3 patients were resistant to these three drugs.

In vitro activities of each cytotoxic drug are shown in Table 4. MTX and Mitomycin-C yielded the highest response rate, with 78% and 73 % response respectively from specimens tested.

In Vitro and In Vivo Chemosensitivity

Correlation between in vitro and in vivo results was available for 16 patients who received chemotherapy (Table 3). Ten of them received preoperative chemotherapy. Among them, only one patient (Case 13) was graded as responder. The other 9 patients were graded as non-responders. Clinical response of the 6 patients who received only postoperative chemotherapy was evaluated by local recurrence and distant metastasis. Five of the 6 patients developed lung metastasis within 24 months after diagnosis. Thus, they were evaluated as non-responders. The other one patient was evaluated as responder.

Table 4 In vitro chemosensitivity of soft tissue sarcoma

Drugs	No. of cases tested	No. of sensitive cases (%)
ADR	26	3 (12)
BLM	25	6(24)
CBP	14	1 (7)
CDDP	26	8 (31)
CPM	26	12 (46)
DACT	25	6 (24)
EPI	13	2 (15)
IFO	26	8 (31)
MMC	26	19 (73)
MTX	26	20 (77)
SM5887	24	2 (8)
THP	24	7 (29)
VCR	26	1 (4)
VP16	14	0 (0)

In vitro and in vivo comparison of chemotherapy resulted in 2 S/S, 10 R/R, 4 S/R, and 0 R/S. The true positive rate (number of S/S) / (number of S/S + S/R) was 33%; true negative rate (number of R/R) / (number of R/S + R/R) was 100%; sensitivity (number of S/S) / (number of S/S + R/S) was 100%; specificity (number of R/R) / (number of S/R + R/R) was 71%; and predictive accuracy (number of S/S + R/R) / (number of S/S + S/R + R/S + R/R) was 75%.

DISCUSSION

Various in vitro Chemosensitivity tests for many kinds of cancers have been reported[6]. Because it is difficult to maintain primary sarcoma cells in culture, the success rate of in vitro assays for sarcoma has generally been low. The clonogenic assay has been studied most extensively and a positive clinical correlation has been found in a variety of tumor types. However the success rate of assays for this sarcoma has been < 20 %[6]. To overcome this problem, we have used early generation tumor xenografts as a cell source of in vitro scintillation assay[8,9]. These xenografts enabled a large number of viable tumor cells to be obtained even from small biopsy specimens. As a result, this system provided a workable assay in 53 % of soft tissue sarcomas tested, which is comparable to that of osteosarcomas in our previous study[8].

An additional advantage of our assay system using tumor xenografts is

that it has prognostic value. The five-year survival rate was significantly lower for the patients with a successful assay (36.6 %) than for those with a failed assay (56.1 %). Patient prognosis is extremely important for the planning and evaluation of therapy. The histological type of soft tissue sarcoma does not always provide sufficient information to predict the clinical course[10]. To date, the histological grade is considered the most important single factor in predicting the survival rate[11,12]. The difficulties in grading sarcomas are interobserver variability and reproducibility[13,14].

Several reports have suggested that positive tumor growth in nude mice is a poor prognostic indicator for the corresponding patient[15-18]. Tumors successfully grown in nude mice are more likely to behave aggressively in patients. However, there has been no previous published report that determined the correlation between tumor engraftment in nude mice and the prognosis of patients with soft tissue sarcoma. In the present study, there was a tendency toward poor prognosis in patients whose tumors successfully grafted in the nude mice. The combination of positive in vivo growth in nude mice and in vitro growth of the tumor may accurately provide a poor prognosis for patients with soft tissue sarcoma.

Clinical correlations of clonogenic assays have been reproducible with 50 % to 70 % true positive prediction and > 90 % true negative prediction[6]. The in vitro chemosensitivity assay shown here has been found to predict resistance of soft tissue sarcoma very accurately: true negative rate of 100 %, which compared favorably to other reports. In contrast, the prediction of sensitivity is low: true positive rate of 33 %, which was even lower than that of osteosarcoma[8]. The system reported here provided a valid tool to determine ineffective drugs in therapy for individual soft tissue sarcoma patients.

There was a lack of response to doxorubicin and ifosfamide in vitro although a significant amount of clinical evidence indicates that these two drugs are the single most effective drugs against soft tissue sarcoma. This paradox was also found in our chemosensitivity assay for osteosarcoma[8]. The efficacy of drug treatment depends on the bioavailability of the intracellular target during drug exposure or on the possibility of the drug reaching the intracellular target in an amount sufficient to exert its cytotoxic effect. The conditions of in vitro drug exposure such as drug exposure time and incubation time in the cell culture medium may not reflect the in vivo conditions. Additional work will be required to determine the accuracy and limitations of the assay.

The authors concluded that the in vitro scintillation assay could be a valuable aid to clinicians in the prognosis of soft tissue sarcoma patients and in

ruling out chemotherapy which would be ineffective. As a result, unnecessary toxic side effect of chemotherapy can be avoided and the quality of life of the patients maintained. In addition, the medical cost could be reduced.

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