Preliminary Study of Chemosenstivity Tests in Osteosarcoma Using a Histoculture Drug Response Assay

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Abstract. *Background: The clinical efficacy of chemotherapy is strongly correlated with the histoculture drug response assay (HDRA) in various types of cancers. However, there have been no previous reports on its use in osteosarcoma. Materials and Methods: Thirty-six fresh samples of human osteosarcoma were evaluated by the HDRA method in order to determine its possible usefulness in the treatment of this cancer. All the materials were obtained either during biopsy or surgical excision at our hospital, between January 2003 and October 2005. Results: A significant inhibition rate in primary biopsies was observed with Adriamycin (ADM) (47.3±15.3%); Cisplatin (CDDP) (36.3±22.3%); and Carboplatin (CBP) (50.5±23.3%); treatment. ADM and CBP demonstrated a statistically significant increase in inhibition rates compared to the other drugs. Conclusion: Even though the HDRA method has many limitations, it might be a feasible and useful technique for selecting and predicting the efficacy of anticancer drugs for osteosarcoma patients.*

There is a general consensus that chemotherapy is essential for the treatment of osteosarcoma. However, individual patient response to chemotherapy varies greatly, even when tumors have been similarly classified. Thus, it would be desirable to perform chemotherapy tailored to the chemosensitivity of specific tumors. Investigators have sought to develop an *in vitro* test to determine which chemotherapeutic agents are effective *in vivo*. However, the attempts to predict tumor response to drugs using *in vitro* cell cultures have failed and, to date, there is no reliable method to select the most efficient chemotherapeutic agents for each type of osteosarcoma. It has been reported that the histoculture drug response assay (HDRA), which is an *in vitro* chemosensitivity test, had a high correlation with clinical response in various types of cancer. However, there have been no previous reports on the use of HDRA in osteosarcoma. In this study, in order to determine the clinical efficacy of an *in vitro* chemosensitivity test in osteosarcoma, freshly biopsied or surgical specimens of osteosarcoma were tested using the HDRA method.

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Materials and Methods

Tested samples. Thirty-six fresh samples of human osteosarcoma were obtained, either during biopsy or surgical excision at our hospital, between January 2003 and October 2005. All the samples were diagnosed as osteosarcoma. Informed consent for this study was obtained from each patient. Based on the history of previous exposure to chemotherapeutic agents, the samples were divided into two groups. Group I comprised twenty initial biopsy samples, from patients who had never been treated with chemotherapeutic agents; Group II comprised sixteen recurrent or metastatic tumor samples, from patients who had previously been treated with chemotherapeutic agents.

Drugs. Ten anticancer drugs were studies: Adriamycin (ADM), Cisplatin (CDDP), Cyclophosphamide (CTX), Ifosfamide (IFS), Mitomycin C (MMC), Bleomycin (BLM), Paclitaxel (Taxol), Dacarbazine (DTIC), Methotrexate (MTX), Etoposide (VP-16), Carboplatin (CBP) and Vincristine (VCR). The cut-off concentrations of these drugs used to distinguish *in vitro* sensitivity and resistance were: ADM 6 μg/ml, CDDP 10 μg/ml, CTX 20 μg/ml, IFS 250 μg/ml, MMC 2 μg/ml, BLM 20 μg/ml, Taxol 75 μg/ml, DTIC 4 μg/ml, MTX 25 ~ 2500 μg/ml, VP-16 50 μg/ml, CBP 100 μg/ml and VCR 2 μg/ml. All the drugs were purchased from Sigma (St. Louis, MO, USA). It was difficult to determine the required concentration of MTX during the *in vitro* study, since a high concentration is needed for the clinical treatment of osteosarcoma. Therefore, it was difficult to verify the experimental value of the MTX concentration.

HDRA. The HDRA was performed according to the previously reported method (1), with slight modification. The tissue was cut into 1- to 2-mm^3 fragments and placed onto 0.5-cm^2 pieces of

collagen sponge-gel (Gel Foam[R]: Pharmacia & Upjohn, UK) in equal quantities. These sponge-gel cultures were then placed into 24-well plates with RPMI 1640 medium (Sigma) containing 20% fetal bovine serum (FBS; U.S. BIIO-TECHNOLOGIES INC., USA) and 100 μg/ml of 100 units/ml penicillin-streptomycin (GIBCO). The anticancer agents were dissolved in RPMI 1640 medium (Sigma) containing 20% FBS; (U.S. BIIO-TECHNOLOGIES INC.) and 100 μg/ml of 100 units/ml penicillin-streptomycin (GIBCO). One ml per well of the solution was poured into a 24-well plate. After incubation for 72 h, 100 μl of 0.06% collagenase (type I; Sigma) solution in HBSS and 100 μl of 0.2% MTT (Sigma) phosphate-buffered saline (PBS) solution, containing 50 mM sodium succinate (Wako Pure Chemical Industries, Tokyo, Japan), were added to each well. After the plates had been incubated for an additional 4 h, the medium was removed and 0.5 ml per well of dimethyl sulfoxide (DMSO) was added to extract MTT-formazan. After 2 h, the extracted solution from each well (100 μl) was moved to a 96-well plate and absorbance was measured with a microplate reader (VERSAmax; Molecular Device, USA) at 540 nm (reference 630 nm).

The inhibition rate was calculated as:

$$\text{Inhibition rate } (\%) = (1 - A/B) \times 100,$$

where A is the mean absorbance of the treated wells per 1 g of tumor and B is the mean absorbance of the control wells per 1 g of tumor. The drug concentration that caused a tumor growth inhibition rate greater than 30% was scored as "sensitive" in the HDRA. This was calculated to compensate for experimental error based on our analysis of the outcome data over an extended period of time.

Statistical analysis. The Wilcoxon Signed Ranks test was used to determine the statistical significance for each drug. A comparison study between the groups was performed with the Mann-Whitney test. A p value of less than 0.05 was considered to be statistically significant. All the data were evaluated by an SPSS program.

Results

Group I was composed of twelve men and eight women, with a mean (± standard deviation) age of 17.5 year (± 9.85 years). The tumors were located on the bones of the extremities, with the most common location being the distal femur (n=10). According to the osteosarcoma subtype, osteoblastic osteosarcoma was the most common (n=13), as noted in Table I. Significant inhibition rates were observed with the use of ADM (47.3±15.3%), CDDP (36.3±22.3%) and CBP (50.5±23.3%). ADM (p=0.039-0.01) and CBP (p=0.01-0.004) caused statistically significantly increased inhibition rates compared to the other drugs (Table II).

Group II was composed of eleven men and five women, with a mean age of 22.4±16.3 years. The most common location was the distal femur (n=10), while the most common subtype was osteoblastic osteosarcoma (n=12), also noted in Table I. The most effective drug was CBP (61.4±17.4%), followed by ADM (60.0±11.9%), CDDP (38.5±19.0%) and VCR (34.3±20.5%). According to the comparison study between Groups I and II, some of the drugs (specifically, ADM, CDDP, DTIC, CTX, VCR and CBP) were more effective in Group II. However, the only statistically significant increase in drug effectiveness occured with ADM treatment (p<0.012).

Table I. *Clinical data of all materials.*

	No.	Gender	Age	Primary site	Subtype
Group I	1	F	10	Distal radius	Telangiectatic
	2	M	11	Distal femur	Osteoblastic
	3	F	10	Distal tibia	Osteoblastic
	4	M	12	Proximal humerus	Osteoblastic
	5	F	16	Distal femur	Osteoblastic
	6	M	52	Distal femur	Osteoblastic
	7	M	13	Proximal humerus	Osteoblastic
	8	F	15	Proximal humerus	Osteoblastic
	9	M	18	Distal femur	Osteoblastic
	10	M	17	Distal femur	Osteoblastic
	11	M	5	Proximal humerus	Osteoblastic
	12	M	24	Proximal tibia	Osteoblastic
	13	F	14	Distal femur	Osteoblastic
	14	F	24	Proximal humerus	Osteoblastic
	15	F	14	Distal femur	Chondroblastic
	16	M	19	Proximal tibia	Chondroblastic
	17	M	15	Distal femur	Chondroblastic
	18	F	20	Distal femur	Chondroblastic
	19	M	14	Distal femur	Fibroblastic
	20	M	25	Proximal femur	Fibroblastic
Group II	21	M	12	Distal femur	Osteoblastic
	22	M	15	Distal femur	Osteoblastic
	23	M	19	Distal femur	Osteoblastic
	24	M	33	Rib	Osteoblastic
	25	F	6	Distal femur	Osteoblastic
	26	F	37	Proximal tibia	Osteoblastic
	27	F	10	Distal femur	Osteoblastic
	28	M	16	Distal femur	Osteoblastic
	29	M	29	Proximal humerus	Osteoblastic
	30	F	13	Distal femur	Osteoblastic
	31	M	63	Proximal tibia	Fibroblastic
	32	F	19	Distal femur	Osteoblastic
	33	M	13	Proximal humerus	Osteoblastic
	34	M	1	Distal femur	Chondroblastic
	35	M	23	Distal femur	Osteoblastic
	36	M	49	Proximal humerus	Chondroblastic

Group I comprised twenty initial biopsy samples, from patients who had never been treated with chemotherapeutic agents. Group II comprised sixteen recurrent or metastatic tumor samples, from patients who had previously been treated with chemotherapeutic agents.

Discussion

In high-grade osteosarcoma, the long-term survival rate has improved over the last 20 years from less than 20% to nearly 80%, with the introduction of adjuvant and neo-adjuvant chemotherapy (2-6). Thus, it is generally accepted that chemotherapy is essential for the treatment of osteosarcoma. Attempts have been made to improve the survival rate further,

Table II. *Chemosensitivity test using HDRA.*

	Inhibition Rate (%)									
Group	MTX	ADR	CDDP*#	IFS*#+	BLM*#	DTIC*+	CTX*	VCR	VP16*	CBP+
I	29.6±21.1	47.3±15.3	36.3±22.3	27.3±12.2	28.0±16.6	19.6±13.7	17.5±12.8	29.5±15.0	26.3±13.4	50.5±23.3
II	21.4±23.7	60.0±11.9	38.5±19.0	21.2±15.1	26.7±17.1	22.3±16.8	19.8±14.9	34.3±20.5	23.5±19.3	61.4±17.4

The data are expressed as means±S.D.
*$p<0.05$, compared to ADR; +$p<0.05$, compared to CDDP; #$p<0.05$, compared to CBP.

through the intensification of chemotherapy or the development of new treatment regimens. A study of the results of chemotherapy trials conducted in many institutions revealed a trend towards improved outcome for patients who had been treated recently with more intensive chemotherapy regimens. However, the intensification and further development of chemotherapy is always limited, because chemotherapeutic doses must be minimal, to avoid adverse side-effects. For safe delivery of intensive chemotherapy, selection of the most effective drugs should also improve the quality of life of individual cancer patients. However, the sensitivity to anticancer drugs differs among cancers, even between those with the same histocultural findings. Drug resistance remains one of the major problems in the treatment of cancer (7, 8). Histological evaluation of the response to chemotherapy is the generally accepted most important prognostic factor in osteosarcoma, but it can be accomplished only after pre-operative therapy, therefore limiting its usefulness in the determination of post-operative therapeutic regimens (9-12). Chemosensitivity testing was used either *in vitro* tumor cell line models or *in vivo* animal models (13, 14). However, a number of difficulties are encountered, due to the limited viability of division and the loss of differentiation characteristics that occur *in situ*. To successfully investigate the pathophysiology of human osteosarcoma, it is necessary to maintain or recreate the characteristic three-dimensional architecture of the tissue in culture. It has been reported that a collagen sponge-gel-supported histoculture from diverse human tumor specimens maintained three-dimensional tissue architecture and function *in vitro* (15, 17). The clinical efficacy of chemotherapy has been shown to be highly correlated with the HDRA data in various cancers (16, 17).

Osteosarcoma treatments currently in use incorporate ADM, CDDP and HDMTX (high-dose MTX), although the optimal chemotherapy regimen remains controversial. The reported response rates for MTX against macroscopic disease vary widely, ranging from no response to 80% (18, 19) and the role of HDMTX in the chemotherapy of osteosarcoma requires further investigation. In this study, MTX evaluation presented certain technical problems, but we plan to determine the optimum concentration required through parallel experiments *in vitro* and *in vivo*. Excluding MTX, the results of this study demonstrated that osteosarcomas were more sensitive to ADM (47.3±15.3%), CDDP (36.3±22.3%) and CBP (50.5±23.3%) than to the other drugs tested. The inhibition rates achieved with ADM and CBP were higher than that of CDDP. CBP has the advantage over CDDP of potentially reduced renal and ototoxicity. However, this agent may be considerably less active against osteosarcoma than CDDP (20), thus limiting its recommendation, although our results indicated that CBP might be useful against osteosarcoma. On evaluation of the sensitivity patterns according to the groups, some drugs (ADM, CDDP, DTIC, CTX, VCR, CBP) achieved more sensitivity in Group II, while the inhibition rate achieved with ADM was significantly increased in recurrent or metastatic osteosarcoma compared to those with a primary diagnosis. Furthermore, intensified high-dose chemotherapy might be needed in patients with recurrent and metastatic disease.

Although, this study had some limitations, mostly related to it being an *in vitro* study, it is the first such chemosensitivity study of osteosarcoma. The outcomes were not analyzed because no attempt was made to correlate these with the chemosensitivity test results. Additional studies are necessary to clarify the role and potential use of HDRA in the treatment of patients with osteosarcoma. Since there is a wide variation in chemosensitivity among osteosarcomas of the same histological classification, the HDRA might be a feasible and useful technique for predicting efficacy and selecting the appropriate anticancer drug for individual patients.

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