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Human tumor clonogenic assay in osteosarcoma for evaluation of clinical efficacy of anti-cancer drugs

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Summary. Twenty-three specimens (biopsied tissue, resected specimens, and resected pulmonary metastases were obtained during biopsy, radical surgery, or thoracotomy from patients with osteosarcoma. Using these specimens, human tumor clonogenic assay (standard HTCA) and cultivated HTCA (HTCA using single-cell suspensions obtained from short-term cultures) were carried out. The percentage of colony-forming ability, which was very small for standard HTCA, increased significantly when cultivated HTCA was performed. With cultivated HTCA, sensitivity tests were possible in 87% of the specimens in contrast to only 28.6% using standard HTCA. We retrospectively analyzed the relationship between the results of HTCA and the clinical efficacy of agents such as methotrexate, adriamycin, cis-platinum, and vincristine, which are frequently prescribed for the treatment of osteosarcoma. This analysis revealed that if drugs are show to be ineffective by this test, they can be regarded as clinically ineffective against that specific tumor target, i. e., the tumor cells are refractory to those drugs. Thus, clinical use of such drugs should be avoided.

Introduction

Osteosarcoma is the most common malignant bone tumor of the extremities. Treatment has undergone marked change – from amputation of the affected extremity in former days to "limb saving" therapy in recent years – mainly due to advances in chemotherapy. However, chemotherapeutic agents can vary in efficacy from case to case. Thus, it has been pointed out that a reliable sensitivity test for anti-caner drugs is necessary.

In recent years, human tumor clonogenic assay (HTCA) has been suggested as a test to evaluate the sensitivity of different human cancer cells to anti-cancer drugs, because it shows a strong correlation with the clinical efficacy of drugs. Although applications of this technique to carcinoma have been reported by many researchers [4, 9], there are few reports on its application to sarcoma. The greatest obstacle to the application of HTCA to sarcoma cells seems to be their low colony-forming activity [4]. Therefore, we recently tried a modified form of a human tumor clonogenic assay, a cultivated assay. Before performing the assay, we incubated the specimens to increase the cell population. Subsequently, we retrospectively compared the results of this sensitivity test and the clinical efficacy of chemotherapy in each case studied.

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Materials and methods

Materials. We used 23 specimens (biopsied tissue, resected primary and pulmonary metastatic lesions) from 15 osteosarcoma cases resected at our hospital during the past 2 years. Biopsied tissue was not obtained from patients treated by chemotherapy, but the other specimens were obtained from patients who had been on chemotherapy to varying degrees (Table 2).

Standard HTCA and cultivated HTCA. Standard HTCA was performed according to the method of Salmon and Hamburger [5], and single-cell suspensions were prepared by enzymatic treatment with collagenase, DNase, and pronase. Cultivated HTCA was performed as follows. First, tumor cells were placed in RPMI-1640 medium (5×10^3 cells/bottle) and incubated until they formed a cell sheet (on average, generally by the first subculture). After collection, cells were treated with the test drug in the same way as in standard HTCA, followed by incubation on the upper layer (plating layer) of a double soft agar medium (5×10^5 cells/plate) at 37 °C and 7% CO_2.

Contact with test drug (Table 1). The sensivity to anti-tumor agents commonly used in chemotherapy of malignant bone and soft tissue tumors (mainly osteosarcoma) was tested. The cultured cells were exposed to the test drug for 1 h at a concentration of one-tenth the maximum blood level found in patients on chemotherapy (i. e., intratumoral level) according to the "drug concentration X time" theory in the Von Hoff experiment [9]. For the time-dependent drug, MTX, the cells were exposed to the agent for 2 weeks. The above experimental conditions were designed according to the plasma-concentration-time product theory ($C \times T$ theory) [9]. Although the concentration of the test drug in the present study was lower than the concentration conventionally used for in vitro evaluation of chemotherapeutic agents, the former has been reported to be closer to the actual intratumoral concentration (i. e., the actual clinical environment) than the latter [1]. Masked compounds such as cyclophosphamide (CPM) and DTIC were used in their activated forms.

Table 1. Antitumor drugs investigated in vitro

Drug	Concentration used in study (μg/ml)[a]
Adriamycin (ADM)	0.04
Aclarubicin (ACR)	0.20
Cis-platinum (CDDP)	0.20
4-hydroperoxy Cyclophosphamide (CPM)	3.00
DTIC	0.10
Ifosphamide	20.00
Vincristine (VCR)	0.01
Methotrexate (MTX)[b]	20.00
Actinomycin D (ACD)	0.005
Mitoxantrone (MXT)	0.02
THP-adriamycin (THP-ADM)	0.50
Mitomycin (MMC)	1.00

[a] Drug concentration chosen as approximately one-tenth the peak plasma concentration in human pharmacokinetic studies
[b] Used as high-dose chemotherapy

Colony counts and evaluation of sensitivity. One week after cell incubation was commenced, clusters consisting of 7 to 50 cells were formed; after 2 weeks, cells formed colonies consisting of more than 50 cells, with a diameter of about 60 μm. At this time, the number of colonies was counted with an automatic particle counter (Hand CP-2000). When 5 or more colonies per plate were found in a non-treated control it was assessed as "plating efficiency present," and if 30 or more colonies were found, it was rated as "sensitivity test possible." The anti-tumor effect of each drug was determined and expressed in terms of inhibition of colony-forming activity in comparison to controls. When a drug reduced colony-forming activity by 70% or more, we assessed the tumor cells as "sensitive" to the drug concerned.

Results

In both standard HTCA and cultivated HTCA, the time needed for colony counts after contact with the test drug was about 2 weeks. However, cultivated HTCA required an extra 9 to 26 days (18.7 days on average) for monolayer culture.

Colony formation with standard and cultivated HTCA (Table 2)

This aspect has already been described in detail [10]. Briefly, in terms of colony forming capacity, cultivated HTCA showed only a slight improvement over standard HTCA. However, in terms of colony counts per inoculated cell, i. e., the plating efficiency, the value recorded with cultivated HTCA (0.0170, almost equivalent to the value obtained for carcinoma) was 4–5 times the value recorded with standard HTCA (0.0039, far below the value recorded for carcinoma). Thus, the "sensitivity test possible" rate was markedly elevated from 28.6% (standard HTCA) to 87.0% (cultivated HTCA). Comparsion of the plating efficiency among specimens revealed that the efficiency was higher for biopsy tissue specimens and pulmonary metastatic lesions, and lower for lesions surgically resected immediately after cehmotherapy. The "sensitivity test possible" rate followed a similar trend.

The correlation between the sensitivity data of these two methods was tested with a chi-square test on the basis of the percent colony inhibition. The relatively high correlation coefficient of $r = 0.7307$ ($P < 0.01$) indicated that almost identical results would be obtained using either of these two methods.

Results of cultivated HTCA (Table 3)

Among various agents used in the chemotherapy of osteosarcoma such as MTX, CDDP, ADM and VCR, we listed agents showing an inhibition rate of between 50% and 70% with cultivated HTCA. Biopsied specimens, which were obtained from patients given no chemotherapy, were most sensitive to MTX and CDDP. A similar trend was also observed in the sensitivity of surgically resected tumor specimens. On the other hand, MTX showed no activity against pulmonary metastatic lesions from patients given long-term MTX chemotherapy, indicating that the tumors had become resistant to the agent. Further, CDDP and ADM showed only low activity against metastatic lesions. In two cases (14, 15) in which most of the tumors had become necrotic due to preoperative chemotherapy, HTCA could not be successfully performed.

Correlation between results of cultivated HTCA and clinical efficacy (Table 4)

We retrospectively evaluated the relationship between the results of cultivated HTCA and the anti-tumor effect of chemotherapy as estimated from the clinical findings (using the classification of Koyama and Saito) or histopathological findings (using the classification of Oboshi and Shimosato) at the time of specimen collection. The following case report is an example of such evaluation.

Case Report

(Case 3) A 48-year-old female with osteosarcoma in the left tibia: Three months after occurrence of initial symptoms, she visited our clinic and osteosarcoma was definitively diagnosed by biopsy. Immediately after one course of high-dose MTX therapy, the patient's leg was amputated above the knee.

Specimens obtained from the amputated leg indicated that tumors had become considerably necrotic due to MTX therapy. Further, cultivated HTCA with the same tumor specimens showed the highest sensitivity to MTX. Thus, we retrospectively estimated that these tumors were sensitive in vitro/sensitive in vivo (S/S: true positive) to MTX. After amputation, the patient was maintained on high-dose MTX therapy; 10 months later, a 7×5 mm metastatic lesion was found in the lung and was resected. Cultivated HTCA with this metastatic lesion showed that the lesion was totally intensitive to MTX and that a colony inhibition of over 50% to 60% was obtained only by treatment with CDDP or VCR. Consequently, we assessed the pulmonary metastatic lesion as resistant in vitro/resistant in vivo (R/R: true negative) to MTX. Based on this finding from cultivated HTCA, MTXtherapy was changed to CDDP and VCR for an additional 6 months. Now, 30 months after the first treatment, the patient is apparently disease-free.

The results of the above-mentioned evaluation are summarized in Table 3. Data for the "sensitivity test possi-

Table 2. Colony growth in osteosarcoma

	Colony-forming ability	Sensitivity test possible		
Method (source)	No. of specimens tested	No. of growth ≧5 colonies/dish[a] %	No. of growth ≧30 colonies/dish %	Plating efficiency[b] (mean ± SD)
Standard assay (fresh tumor)	21	15 (71.4)	6 (28.6)	0.0039±0.0025
Cultivated assay (cultured tumor cells)	23	21 (91.3)	20 (87.0)	0.0170±0.0134
from biopsy	6	6 (100)	5 (83.3)	0.0188±0.0149
from surgery	9	7 (77.8)	7 (77.8)	0.0071±0.0026
from metastases	8	8 (100)	8 (100)	0.0203±0.0111

[a] 5×10^5 cells plated per dish
[b] Number of colonies / number of nucleated cells × 100%

Table 3. Effects of sensitivity tests according to cultivated HTCA of osteosarcoma

Cases	Biopsy	Primary tumor	Lung metastasis
1.17/F			CDDP>VCR>ACR (S/R(CDDP))
2.15/M			VCR>CDDP>ACR (R/R(MTX))
3.48/F		MTX>ADM>VCR (S/S(MTX))	-->CDDP>VCR (R/R(MTX))
4.12/M	CDDP>MTX>VCR	CDDP>ADM>MTX (S/S(MTX))	
5.50/M	MTX>ADM>CDDP		
6.21/M		ACR>ADM>VCR (R/S(MTX))	-->MMC>CPM (R/R(CDDP))
7.13/M	CDDP>ADM>ACR	CDDP>ADM>-- (R/R(MTX))	
8.7/F	CDDP>VCR>ACR	CDDP>ACR>VCR (R/R(MTX))	
9.11/M	MTX>CDDP>VCR	MTX>CDDP>VCR (S/S(MTX))	
10.8/M	Failed		
11.21/M		MTX>CDDP>ADM (S/S(MTX))	-->CPM>ACD (R/R(CDDP))
			-->CPM>ADM (R/R(CDDP))
12.16/M			CDDP>-->ADM (R/R(MTX))
13.16/F			CDDP>ADM>MTX (S/R(MTX))
14.14/M		Failed	
15.38/M		Failed	

Table 4. In vitro (cultivated HTCA) and in vivo (clinical effect) associations by retrospective evaluation

In vivo \ In vitro	Sensitive (S)	Resistant (R)
Sensitive (S)	▲▲▲▲	▲
Resistant (R)	●▲	▲▲●▲▲ ●●▲
		▲ MTX ● CDDP

Materials	S/S	S/R	R/S	R/R
15	4	2	1	8

True positive rate (sensitivity index) = 4/6 = 66.7%
True negative rate (resistance index) = 8/9 = 88.9%
False-positive: 2/6 = 33.3%
False-negative: 1/9 = 11%

ble" cases are summarized in Table 4. Four cases were rated as S/S (true positive), eight cases as R/R (true negative), two cases as false-positive and one case as false-negative. The true positive and negative rates consisted of 66.7% and 88.9% of the total, respectively, thus indicating that the results of cultivated HTCA correlated highly with retrospectively evaluated clinical efficacy.

Discussion

Applications of cultivated HTCA to osteosarcoma

Methods for testing the sensitivity of malignant bone tumor in the extremities to anti-cancer agents have been studied at our department for a considerable time. These methods included observation of degenerative changes in cultured human osteosarcoma cells after stimulation by test drugs [2]; cell kinetic analysis using autoradiography or flow cytometry [8]; a study using ^{89}Sr-induced osteosarcoma [6], nude mice or subrenal capsular transplantation [3]. However, none has yet been applied clinically.

HTCA, which uses a sophisticated culture method and a double soft agar medium, promotes colony formation and at the same time excludes fibroblasts. This approach, made on the assumption of the existence of a tumor stem cell, is receiving much attention because of its good correlation with the clinical efficacy of drugs in carcinoma (Salmon and Hamburger [5, 9]). In sarcoma cases, however, applications of this technique reported in the literature are very rare due to the low plating efficiency of sarcoma cells under the experimental conditions generally used.

Based on our experience [2] that osteosarcoma can be consecutively incubated for at least several months, we realized that plating efficiency can be elevated by this culture technique. Further, preliminary experiments demon-

strated that sensitivity tests with cultured HTCA of one or two passages yields almost the same results as those obtained from standard HTCA [7, 10]. Although there are some questions about this approach (e. g., the period of culture before evaluation), we consider this to be a useful technique.

Specimens

The fact that no great difference was noted in terms of sensitivity to the test drug between biopsy tissue specimens from patients who had not undergone chemotherapy and resected tumors in these who had chemotherapy indicates that tumor sensitivity does not change significantly after one or two cycles of chemotherapy. This was also true in the study on patient tissue that had shown the efficacy of chemotherapy, thus indicating that cells that have survived one or two cycles of chemotherapy have undergone little change in character.

Resected tumors and pulmonary metastatic lesions were clearly different in terms of sensitivity, indicating that the most resistant tumor cells were gradually selected during the course of repeated chemotherapy, and that these formed and maintained metastatic lesions. Conversely speaking, the above finding suggests the possibility that of a large number of pulmonary micrometastases, the chemotherapy-sensitive ones disappeared in the course of chemotherapy.

Results of the present technique and its clinical applications

Although data yielded by a retrospective evaluation may be more subject to question than a prospective randomized experiment, the former type of investigation is inevitable at the present stage of the technique, and we believe our results are significant. Moreover, the results of sensitivity tests using this technique significantly correlated with clinical efficacy. Furthermore, drugs assessed as non-active by the above technique have a high probability of being clinically ineffective, which can indicate that such drugs showed be replaced by others. This finding is significant in as much as it is a first step from the experimental stage to the stage of practical application for the use of an anticancer agent sensitivity test in osteosarcoma. In particular, in the case of osteosarcoma, commonly used agents such as MTX, CDDP, and ADM can have significantly morbid side effects; thus ineffective administration of these agent should be avoided. For this reason, the present technique may play a role as a useful chemotherapy "navigator" [7]. Furthermore, the results of the present study suggest that some tumors acquire resistance to drugs in the course of long-term chemotherapy, and that switching to other drugs with different anti-cancer mechanisms and spectra may be required during routine chemotherapeutic treatment of patients suffering from osteosarcoma.

References

1. Alberts DS, Chen HSG, Salmon SE (1980) In vitro drug assay; pharmacologic considerations. In: Cloning of human tumor stem cells. Alan R. Liss, New York, p 197
2. Manabe S, Takase B, Miaki H, Yamauchi S, Tomita K (1975) Screening test of anti-cancer agents in vivo using heterotransplantation of cultured human bone tumor cells. Jpn J Cancer Clin 21: 599 (in Japanese)
3. Munehiro C (1982) Screening test of anticancer drugs on human osteosarcoma heterotransplanted in nude mice. Int Orthop 6: 181
4. Salmon SE (1984) Human tumor colony assay and chemosensitivity testing. Cancer Treat Res 68: 117
5. Salmon SE, Hamburger AW, Soehlen BGM, Alberts DS, Moon TE (1978) Quantitation of differential sensitivity of human tumor stem cells to anticancer drugs. N Engl J Med 298: 1321
6. Tomita K (1985) Experimental studies on high-dose methotrexate with citrovorum factor chemotherapy for ^{89}Sr-induced osteosarcoma. Prog Med 5: 2218
7. Tomita K (1985) Chemosensitivity test in osteosarcoma. Rinsho Seikei Geka 20: 1305 (in Japanese)
8. Tomita K, Plager JE (1979) In vivo cell cycle synchronization of the murine sarcoma 180 tumor following alternating periods of hydroxyurea blockade and release. Cancer Res 39: 4407
9. Von Hoff DD, Casper J, Bradley E, Sandbach J, Jones D, Makuch R (1981) Association between human tumor colony-forming assay result and response of an individual patient's tumor to chemotherapy. Am J Med 70: 1027
10. Yokogawa A (1985) An experimental study on tumor colony forming assay of osteosarcoma. J Juzen Med Soc 94: 744 (in Japanese)

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