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Originals

A Trial of the in vitro Sensitivity Test of Anti-Cancer Drugs Using Primary Cultured Urogenital Cancer Cells

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Summary. An in vitro chemosensitivity test was applied to clinical specimens of urogenital cancer tissues obtained at operation. Incorporation of ^{3}H-leucine into primary cultured cells 24 h after treatment with cytotoxic drugs was used as an index for cell viability. Primary cell culture was performed using specimens obtained from 37 patients including 20 with transitional cell carcinoma, 15 with renal cell carcinoma and 2 with testicular cancer. Primary cell growth was achieved in 27 (73%) out of 37 specimens and 10 were tested for chemosensitivity. Each specimen of the tumor revealed different sensitivity to drugs, and results of quadruplicate tests for each specimen were identical. It was concluded that the present method of measuring incorporation of radioactivity using urogenital cancer cells primarily cultured in microtiter plate is practically applicable to an in vitro chemosensitivity test.

Key words: Chemosensitivity test, Primary cell culture, Anti-cancer drugs.

Introduction

Many anti-cancer drugs have been used in either the prophylactic or the therapeutic treatment of patients with cancer. If possible, it is clinically of great value to select effective anti-cancer agents prior to initiation of the treatment. Several trials have been performed for this purpose using cancer tissues or cells directly obtained from patients. The in vitro assay procedure proposed by Shrivastav et al [9] used cancer cells cultured on 96-well microtiter plate and measured drug induced inhibition of incorporation of radiolabeled precursor into DNA, RNA and protein. We have tried to simplify their method and have applied it to several human urogenital malignancies. The possibility of the clinical application of the assay has been investigated.

Materials and Methods

Materials. Thirty-seven patients selected for the present study had urogenital tumors and had received neither chemotherapy nor radiotherapy; there were 20 cases with transitional cell carcinoma, 15 with renal cell carcinoma and 2 with testicular carcinoma. T-24 cells, an established cell line of human transitional cell carcinoma, were used for control studies.

Preparation of Tumor Cells. Immediately after resection, a portion of the tumor was placed in an ice-cold medium of Ham's F12 solution containing 8% fetal calf serum and 100 μg/ml kanamycin (basal medium). After removing normal and necrotic tissues, the tumor tissue was cut into small pieces, washed with Hanks' solution and digested at 37 °C for 2 h with 0.2% type IV collagenase disolved in the basal medium. The resultant dispersed cell supsension was filtered through an #80 platinum mesh and passed through a fine needle of 26 and 1/2 gage. The cells were collected by centrifugation at 200 x g for 5 min and resuspended in the basal medium supplemented with 50 ng/ml epidermal growth factor (complete medium). Viable cell number was counted by a hemocytometer after trypan blue staining.

Tumor cell culture and in vitro chemosensitivity test: Each primary tumor cell suspension was adjusted to 2×10^4 cells/ml in the medium unless otherwise mentioned. 0.1 ml of the adjusted cell preparation was placed in a well of a microtiter plate and cells were cultured for 4 to 5 days and cells were actively growing and forming semiconfluent monolayers in the well of microtiter plates. Then, each anti-cancer agent was added in quadruplicate at three different concentrations and was incubated for 2 h. Drugs were washed twice with Hanks' solution and were cultured for another 24 h in the fresh medium. Then 1.0 μCi of a tritiated compound in 0.1 ml complete medium was added to each well. After additional incubation for 2 h, the cells of each well were separately collected and the radioactivity in the cells was determined. Cells were judged as sensitive to an agent when incorporation of radioactivity was reduced to 10% of control by the treatment with it.

Chemicals. Trypsin, Ham's F12 and Hanks' balanced solution were purchased from Gibco Lab. (Grand Island, N.Y.), Kanamycin and type IV collagenase were obtained from Sigma Chem. Co. (St. Louis, MO). Fetal calf serum and epidermal growth factor were products of Armour Pharmacent Co. (Phoenix, Arizona) and Collaborative Res. Inc. (Waltham, Mass.).

Anti-cancer agents tested were doxorubicin hydrochloride (ADM: Kyowa Hakko Kogyo Co., Tokyo), carboquone (CQ: Sankyo Co.,

Table 1. Summary of primary cultures

Tumors	Number of cases	
	cultured	tumor cell growth
Transitional cell	20[a]	13[b] (65%)
Renal	15	13[c] (87%)
Testicular	2	1
Total	34	27 (73%)

[a] Bacterial contamination occurred in 4 cases
[b,c] Fibroblasts became predominant in 3 and 2 cases, respectively

Tokyo), cis-diamine dichloroplatinum (CDDP: Bristol-Myers K.K., Tokyo) and aclarubicin hydrochloride (ACR: Sanraku Ocean Co., Oosaka), bleomycin (BLM: Nihon Kayaku Co., Tokyo.). They were dissolved in the complete medium and stored as a stock solution at −70 °C until use. All other reagents are of analytical grade.

Results

Primary cell growth of tumor was judged on the basis of morphological features of cells and colonies formed in the microtiter plate, at 4 or 5 days. As shown in Table 1, primary cell growth was observed in 13 (65%) of 20 patients with transitional cell carcinoma, in 13 (87%) of 15 patients with renal adenocarcinoma and one of 2 patients with testicular cancer. Primary cell culture failed in 4 cases of transitional cell carcinoma because of bacterial contamination. In 3 cases of transitional cell carcinoma and 2 cases of renal adenocarcinoma, the growth of tumor cells was poor.

Incorporation of radioactive precursors into primarily cultured cells: In order to find an appropriate index to represent the growth status of cultured cells, incorporation of ^{3}H-tyhmidine, ^{3}H-uridine and ^{3}H-leucine into primarily cultured cells was examined and compared with that into an established bladder cancer cell line, T-24 (Fig. 1). Although incorporation of radioactivity was not as extensive in primarily cultured cells as in T-24 cells, the incorporated radioactivity orginated from ^{3}H-uridine and ^{3}H-leucine increased with culture time at least until 6 days. Whereas, the incorporation of ^{3}H-tyhmidine into primary cultured cells was maintained in low level during the whole course of the culture. Usually, the number of tumor cells prepared for culture from an operative specimen was too limited to examine the incorporation of two compounds. And, in order to simplify the assay, ^{3}H-leucine was used for the following study.

Chemosensitivity test concerned the following drugs CQ, ADM, ACR, BLM and CDDP. ADM was examined for 5 cases of transitional cell carcinoma, 3 cases of renal adenocarcinoma and one case of testicular cancer. The sensitivity varied widely from one specimen to an other as shown in Fig. 2. CDDP was examined for 7 primary cell cultures including 3 cases of transitional cell carcinoma, 3 cases of renal adenocarcinoma and one case of testicular cancer. Two cultures, one from renal adenocarcinoma and the other from testicular cancer were sensitive to CDDP at 100 μg/ml (Fig. 3). Chemosensitivity in vitro to CQ was examined using 10 primary cell cultures including 6 transitional cell carcinoma, 3 renal adenocarcinoma and one testicular cancer. As shown in Fig. 4, almost all tumors except for one revealed sensitivity to CQ at 10 μg/ml. Figure 5 shows the sensitivity of 6 cultures to ACR, including 4 transitional cell carcinoma and 2 renal adenocarcinoma. Inhibition of

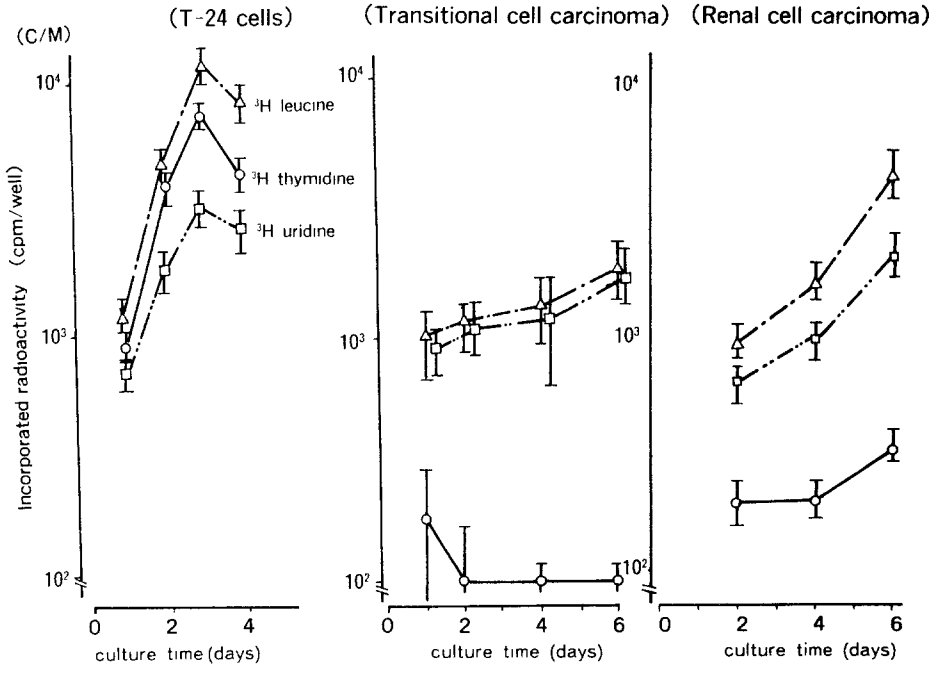
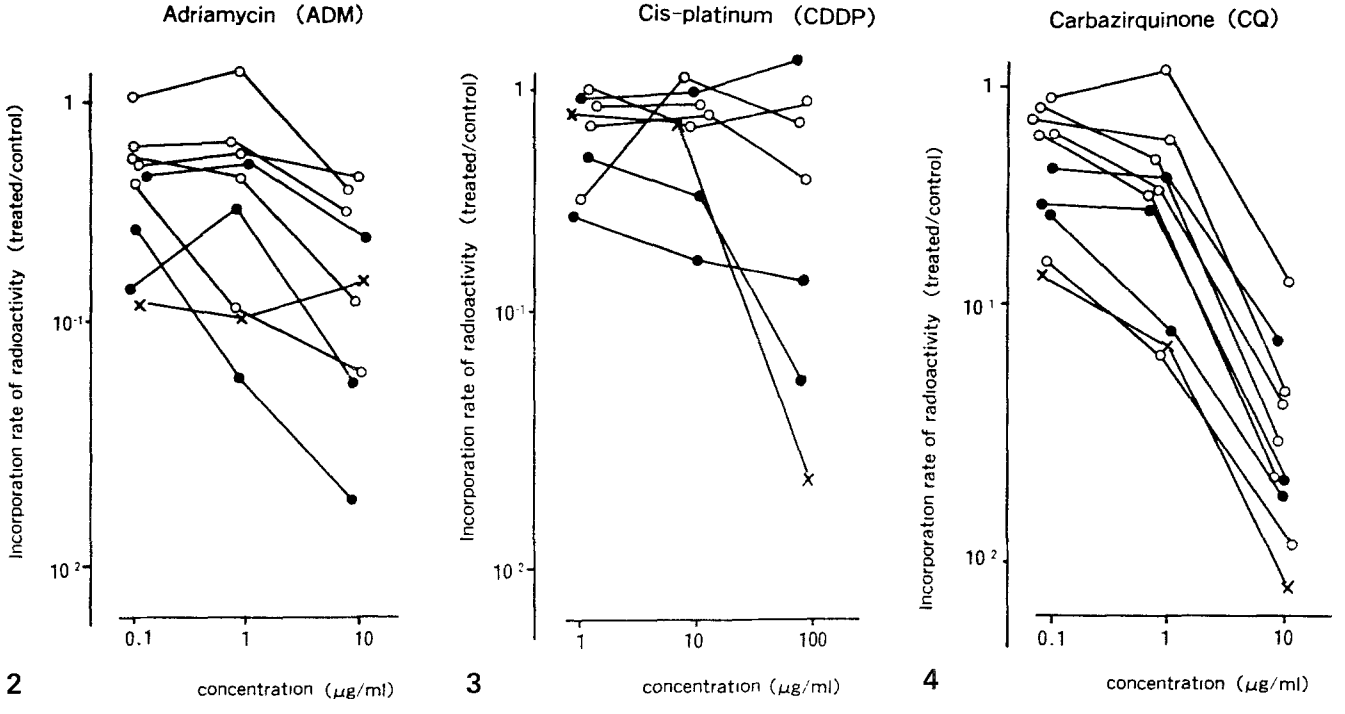


Fig. 1. Incorporation of radioactive precursors into cells of either primary cultured or an established cell-line, T-24: Tumor cells (1×10^4 cells/well) were incubated with eigher tritiated thymidine (– ○ –), uridine (– □ –) or leucine (– △ –). Each symbol in the figure represents the mean of 12 cultures ± S.E. Incorporation of each radioactive compoiund into T-24 cells (*panel A*) rapidly increased and reached maximum on culture day 3. In contrast, in primary cultured cells of transitional cell carcinoma (*panel B*) and renal adenocarcinoma (*panel C*), the increase of incorporated ^{3}H-uridine and ^{3}H-leucine was linear but slow and incorporation of ^{3}H-thymidine was very limited throughout culture for 6 days



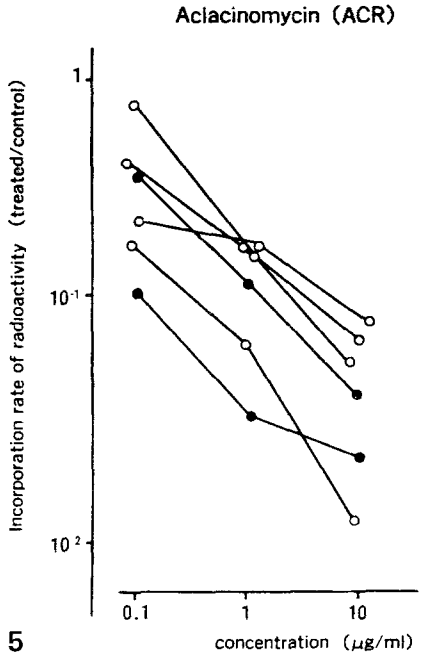


Fig. 2. Effect of doxorubicin hydrochloride (ADM) on the incorporation of ^{3}H-leucine into primarily cultured cells. Incorporation rate is the ratio of radioactivities incorporated into cells treated with drugs to those into cells without the treatment of drugs. The incorporation of ^{3}H-leucine decreased below 10%, of controls in two cases of renal adenocarcinoma (– • –) and in one case of transitional cell carcinoma (– ○ –) in the presence of 10 μg/ml of ADM, and in one case of renal adenocarcinoma in the presence of 1 μg/ml of ADM

Fig. 3. Effect of cis-dichloroplatinum (CDDP) on ^{3}H-leucine incorporation. One culture from renal adenocarcinoma (– • –) and another culture from testicular cancer (– x –) were sensitive to CDDP at 100 μg/ml but others showed poor response. Refer to the text and the legend to Fig. 2 for further details

Fig. 4. Effect of carboquone (CQ) on ^{3}H-leucine incorporation. Three strains of cells including a transitional cell carcinoma (– ○ –), a renal adenocarcinoma (– • –) and a testicular cancer (– x –) were sensitive to CQ at 1 μg/ml. Almost all tumor cells except for a case of transitional cell carcinoma revealed sensitivity to CQ at 10 μg/ml

Fig. 5. Effect of aclarubicin hydrochloride on ^{3}H-leucine incorporation. The incorporation was inhibited in all cultures by ACR at 10 μg/ml of ACR. One culture from transitional cell carcinoma (– ○ –) and one from renal adenocarcinoma (– • –) were sensitive to ACR even at 1 μg/ml

radioactivity incorporation was observed in three specimens after treatment with ACR at 10 μg/ml. Effects of cytotoxic agents on primarily cultured cells are shown in Fig. 6. CQ was effective on primarily cultured cells from transitional cell carcinoma, renal adenocarcinoma and testicular cancer, while CDDP and BLM were effective only on primarily cultured cells from testicular cancer.

Discussion

In the present study, the in vitro chemosensitivity test using primarily cultured cancer cells in a microtiter plate and the incorporation of ^{3}H-leucine as an index of cytotoxicity of anticancer drugs were applied for several urogenital malignancies and the effect of several anti-cancer drugs on these cells were investigated.

In culture, tumor cells are the predominant strain which forms monolayer colony at the initial 5 or 6 days of our culture method. Fibroblasts and other cells increase during culture and can be observed by microscopic examination. Cancer cells could be selected from tumor specimens and then used for the chemosensitivity test. In the present study, primary cell growth was obtained in 27 (73%) out of 37 patients. Some of the failures, especially in culture of transitional cell carcinoma were caused by bacterial contamina-

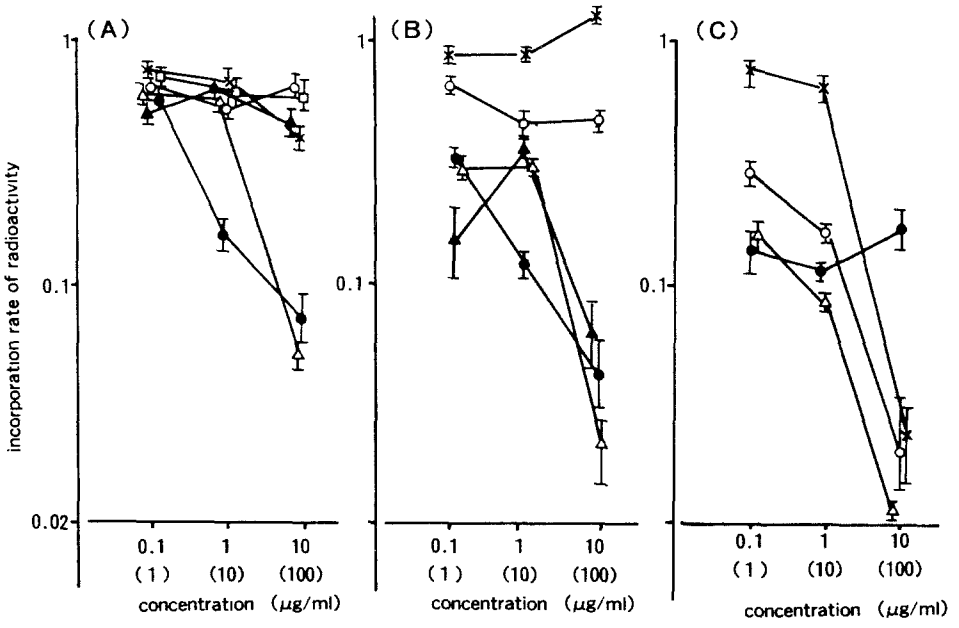


Fig. 6. Effects of in vitro chemosensitivity test. A case of transitional cell carcinoma (*panel A*) was sensitive to CQ (– △ –) and ACR (– ● –) at 10 μg/ml. A case of renal adenocarcinoma (*panel B*) was sensitive CQ, ACR, and ADM (– ▲ –) at 10 μg/ml. A case of testicular cancer was sensitive to CDDP (– ○ –) at 100 μg/ml CQ and BLM (– x –) at 10 μg/ml

tion although anti-biotics had been added to the culture medium. In most cases of other failure, the number of viable tumor cells prepared from operative specimens were not enough for testing.

In our preliminary study [7] it was shown that sensitivity of HeLa S_3 and T-24 cells to alkylating and antibiotic anticancer agents could be reliably tested by utilizing the culture system in a microtiter plate and by the incorporation of radioactive compounds into cells as an index of living cells. The time study for primary cell culture revealed that incorporation of tritiated uridine and leucine and of thymidine correlated well with proliferation of cells in culture. Therefore, incorporation of ^{3}H-leucine was used for an index of cell viability after the treatment of cells with anticancer drugs [3]. Based on the results of the previous study [7] the incorporation was tested 24 h after the treatment with drugs. Kato [5] also reported ^{3}H-leucine incorporation as a reliable measure for in vitro assay system on the basis of the result of a cell kinetic study. Thus the choice of the incorporation of ^{3}H-leucine as an index using primarily cultured cells in a microtiter plate could make the assay sensitive enough for testing with a small number of cells obtained from solid tumors.

Recently, a stem cell assay by Salmon and associates [8] has been used with a good correlation between in vitro and in vivo responses [4], but one of the technical problems noticed inperforming the assay [2, 6] is that the method requires 3 to 4 weeks to obtain results. Our present test needs only 6–8 days to show results. Furthermore, the present test is not labor intensive though, more technical improvement is required for the clinical application of this chemosensitivity test.

In the present study, effects of cytotoxic agents, CQ, ADM, ACR, BLM and CDDP on tumor cells differed with specimens of the same kind of tumors as well as with different kinds of urogenital tumors. For example, BLM and CDDP were effective on primarily cultured cells from testicular cancer but not effective on other tumors. This different sensitivity of tumor cells to various drugs in vitro could depend upon the different sensitivity of tumor in vivo. The correlations between the in vivo and in vitro responses are suitable for further study. The present study, however, indicates that the in vitro chemosensitivity test using primary culture of cancer cells in a microtiter plate and incorporation of ^{3}H-leucine might be applicable to clinical use because of its simplicity, reproducibility and short test time, as described by Day et al. and Shrivastav et al. [1, 9].

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