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Chemo-radionuclide therapy for thyroid cancer: Initial experimental study with cultured cells*

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Radioiodine therapy has long been used for distant metastases of thyroid cancer. Although partially effective in most cases, it can render a complete cure only in a limited number of patients. One way to enhance its efficacy would be to combine it with antineoplastic agents. Here we describe an initial *in vitro* evaluation with 4 thyroid cancer cell lines. ***Methods:*** Cells were sparsely seeded in microtiter plates and allowed to grow for 2 days; then they were exposed to sublethal concentrations of cisplatin (CDDP), doxorubicin (Dox), or 5-fluorouracil (5-FU), followed by treatment with I-131 for 48 hr. Cell survival was measured with a commercial kit based on the colorimetry of succinate dehydrogenase activity. ***Results:*** Chemotherapeutic drugs exerted similar concentration-dependent cytotoxic effects in all 4 cell lines. The doses necessary to reduce the surviving fraction to half of the control were about 3 μg/m*l* for CDDP, 0.3 μg/m*l* for Dox, and 3 μg/m*l* for 5-FU (when used continuously for 48 hours). On the other hand, sensitivity to I-131 irradiation differed among the lines; same doses (7.4–14.8 MBq/m*l*) caused the greatest damage in FRO cells, a modest effect in NPA and WRO, and only minimal change in B-CPAP. The combined effect was most demonstrable in wells treated with Dox and radioiodine, whereas the addition of CDDP or 5-FU had marginal or insignificant merit, respectively. In FRO cells, half-lethal doses of the above mentioned CDDP, Dox, and 5-FU, when used together with 14.8 MBq/m*l* I-131, reduced cell survival to 54.5%, 29.4% and 33.4%, respectively, vs. 60.2% with radioiodine alone. ***Conclusion:*** *In vitro*, clinical concentrations of Dox can accelerate the killing of thyroid cancer cells by radioiodine. These favorable experimental results warrant future studies to evaluate whether this new bidisciplinary approach is clinically relevant and feasible.

Key words: thyroid cancer, radioactive iodine, radionuclide therapy, cancer chemotherapy, doxorubicin

INTRODUCTION

RADIOACTIVE IODINE (RAI) therapy has been used for decades for distant metastases of differentiated thyroid cancer (DTC). Complete remission with this mode of treatment can be expected in younger patients with miliary or micronodular pulmonary lesions.[1,2] But large skeletal masses or macronodular lung metastases, often seen in older subjects, show relative resistance to RAI with the usual dose ranges of 4–6 GBq, even in cases with scintigraphically significant uptake in these lesions, probably due to uneven distribution of the radionuclide and an insufficient radiation dose delivered. Giving a very high dose (more than 10 GBq) of RAI to patients[3] to treat these latter lesions might improve therapeutic efficacy, but excessive dose escalation will carry the risk of serious side effects such as bone marrow toxicity and lung fibrosis, in addition to the increased environmental burden of contaminated waste and sewage in internal radiation facilities. As an alternative, we came up with an idea for

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regular-dose RAI therapy potentiated with antineoplastic drugs such as cisplatin (CDDP), doxorubicin hydrochloride (Dox), or 5-fluorouracil (5-FU), which is in principle analogous to the combination therapy already practiced widely in the field of external irradiation for various cancers.[4–6] That "chemoradiation" take advantage of enhanced sensitivity of tumor cells to ionizing radiation via a variety of mechanisms which the drugs can exert in concentrations less than their optimal cytotoxic ranges.[5] On top of these known radiosensitizing effects, the addition of tumoricidal drug may also help eliminate de-differentiated clones within metastatic lesions of DTC which show insufficient uptake of RAI, as suggested by an anecdotal conversion of radionuclide-refractory metastases to iodine-concentrating ones after chemotherapy.[7] Furthermore, an experimental report implies that some chemicals possibly restore lost expression of sodium-iodine symporter in de-differentiated thyroid carcinoma.[8]

This communication describes initial, basic *in vitro* studies of concomitant chemoradionuclide therapy with monolayer culture of 4 well-characterized thyroid cancer cell lines and clinically used drugs.

MATERIALS AND METHODS

Materials: Plastic culture ware was purchased from Iwaki Glass Co. (Funabashi, Japan); RPMI-1640 culture medium without phenol red, penicillin, streptomycin, trypsin from porcine pancreas, ethylene-diamine tetra-acetate (EDTA), CDDP, Dox, and 5-FU from Sigma

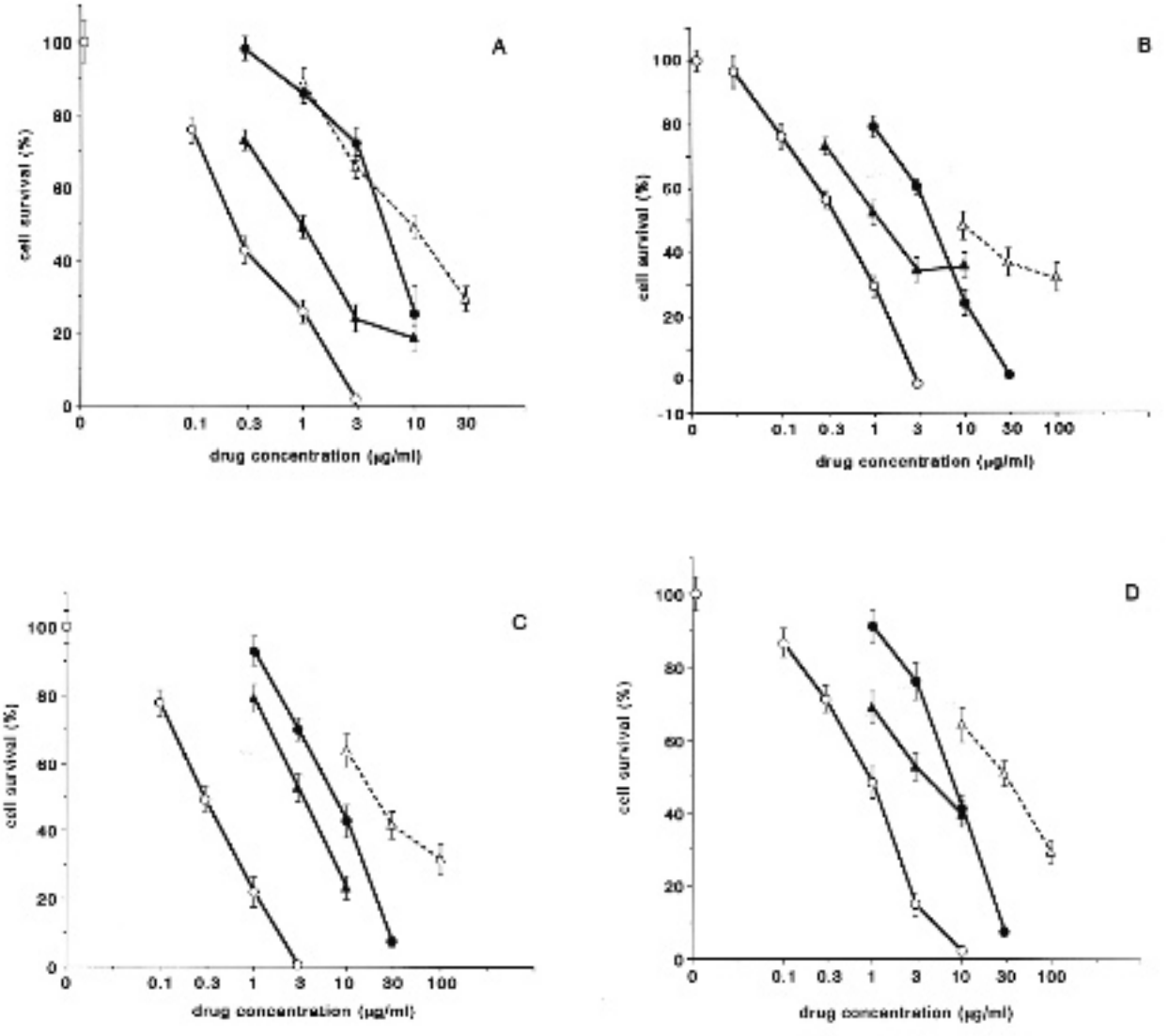

Fig. 1 Effect of the 3 antineoplastic agents on survival of thyroid cancer cells. A: FRO, B: NPA, C: WRO, D: B-CPAP. ● = cisplatin, ○ = doxorubicin, ▲ = 5-fluorouracil (48 hr incubation), △ = 5-fluorouracil (4 hr incubation). Bars represent standard deviation of quadriplicate samples.

Chemical Co. (St. Louis, MO); fetal calf serum (FCS) from GIBCO-BRL (Gland Island, NY); carrier-free NaI-131 from DuPont-New England Nuclear (Boston, MA); a cell-counting kit consisting of WST-1 dye and 1-methoxy-PMS solvent from DOJINDO (Kumamoto, Japan).

Cell culture: Thyroid cancer cell lines FRO, NPA and WRO were established in UCLA,[9,10] originaly from human specimens of anaplastic, papillary and follicular histopathology, respectively. B-CPAP was immortalized in CNRS[11] from papillary carcinoma tissue and obtained through DSM (German Collection of Microorganism and Cell Cultures, Braunschweig, Germany). Cells were routinely maintained in 100 mm culture dishes in RPMI-1640 supplemented with 10% FCS and antibiotics (penicillin 50 μg/m*l* and streptomycin 50 μg/m*l*). For cytotoxicity tests, they were dispersed with 0.05% trypsin and 0.05% EDTA in phosphate-buffered saline (PBS, pH 7.2) and seeded in flat-bottom 96-well microculture plates in 50 μ*l*/well of the medium. Depending on the doubling time of each line, cell density at seeding was adjusted (range 100–2000 cells/well) so that control wells without a cytotoxic or radioactive reagent could reach subconfluence at the measurement of the survival rate as described below.

Cytotoxicity assay: To measure cell survival, we employed a commercial cell-counting kit based on colorimetry of succinate dehydrogenase activity. As a member of a cellular energy-generating system, this enzyme is ubiquitous in all kinds of cells and active only in living ones. The underlying principle of the kit is the same as the MTT assay developed by Mosmann[12] and modified by Denizot and Lang,[13] but by using a new tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate sodium (WST-1), the new test does not require dissolving the resultant formazan crystals.[14]

Two days after inoculation of the cancer cells, the culture supernatant was removed and replaced with fresh medium containing various concentrations of antineoplastic agents. After 4 hr the wells were washed and then replenished with 100 μ*l*/well of new medium containing NaI-131 (0–14.8 MBq/m*l*). A higher concentration could not be used due to the safety regulation of the institution, which set a daily limit on the amount of each radionuclide in laboratories. In some experiments the replaced medium also contained antineoplastic agents at the same concentrations as used for short-term exposure besides RAI. Forty-eight hours later, the culture medium was aspirated, and cells were incubated with 100 μ*l*/well of WST-1 solution, reconstituted with 1-methoxy-PMS as instructed and then diluted with 10 times the volume of PRMI-1640, at 37°C for 2 hours.[13,14] The plates were read at a wavelength of 405 nm for formazan, with 630 nm as a reference wavelength, in a multiwell spectrophotometer (model SME-3400, Iwaki Glass). Wells containing medium but not cells served as blanks. Assays were done in triplicate or quadriplicate, and the same kinds of experiments were repeated at least 4 times to confirm the results. To rule out significant effect of cross-radiation from neighboring wells, the geometric assignment of points containing radioiodine was changed in each repeat study. Results of the colorimetric assay were always in good agreement with microscopic inspection of the culture plates.

Statistics: Significance of difference between data points was evaluated by ANOVA (analysis of variance) test.

RESULTS

Effects of antineoplastic agents alone: As shown in Figure 1A–D, CDDP, Dox and 5-FU showed signs of dose-dependent inhibition of growth comparable in all four cell lines. Short-term (4 hr) exposure to 5-FU was much less cytotoxic than the continuous treatment, needing approximately 10-times higher concentrations to achieve similar cell killing (dotted line in Fig. 1A–D). CDDP and Dox showed no signs of such time-dependency, acting similarly in either short- or long-term incubation (data not shown). From these curves we estimated that the concentrations necessary for 50% growth inhibition are about 3 μg/m*l* for CDDP, 0.3 μg/m*l* for Dox and 3 μg/m*l* for 5-FU (when used continuously for 48 hours).

Effects of radioiodine alone and combined with antineoplastic drugs: As shown in Figure 2, I-131 dose-dependently inhibits proliferation of FRO, NPA, WRO and B-CAP cells, although the extent varied widely among the lines; same doses (7.4–14.8 MBq/m*l*) caused the greatest damage in FRO cells, had a modest effect in NPA

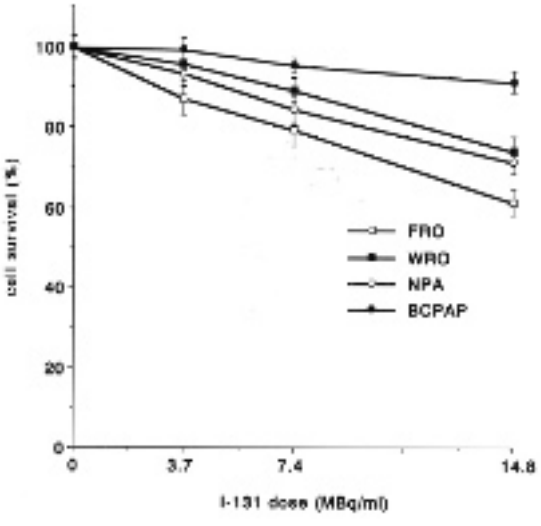

Fig. 2 Effect of I-131 irradiation (for 48 hr) alone on survival of cancer cells. Bars represent standard deviation of quadriplicate samples.

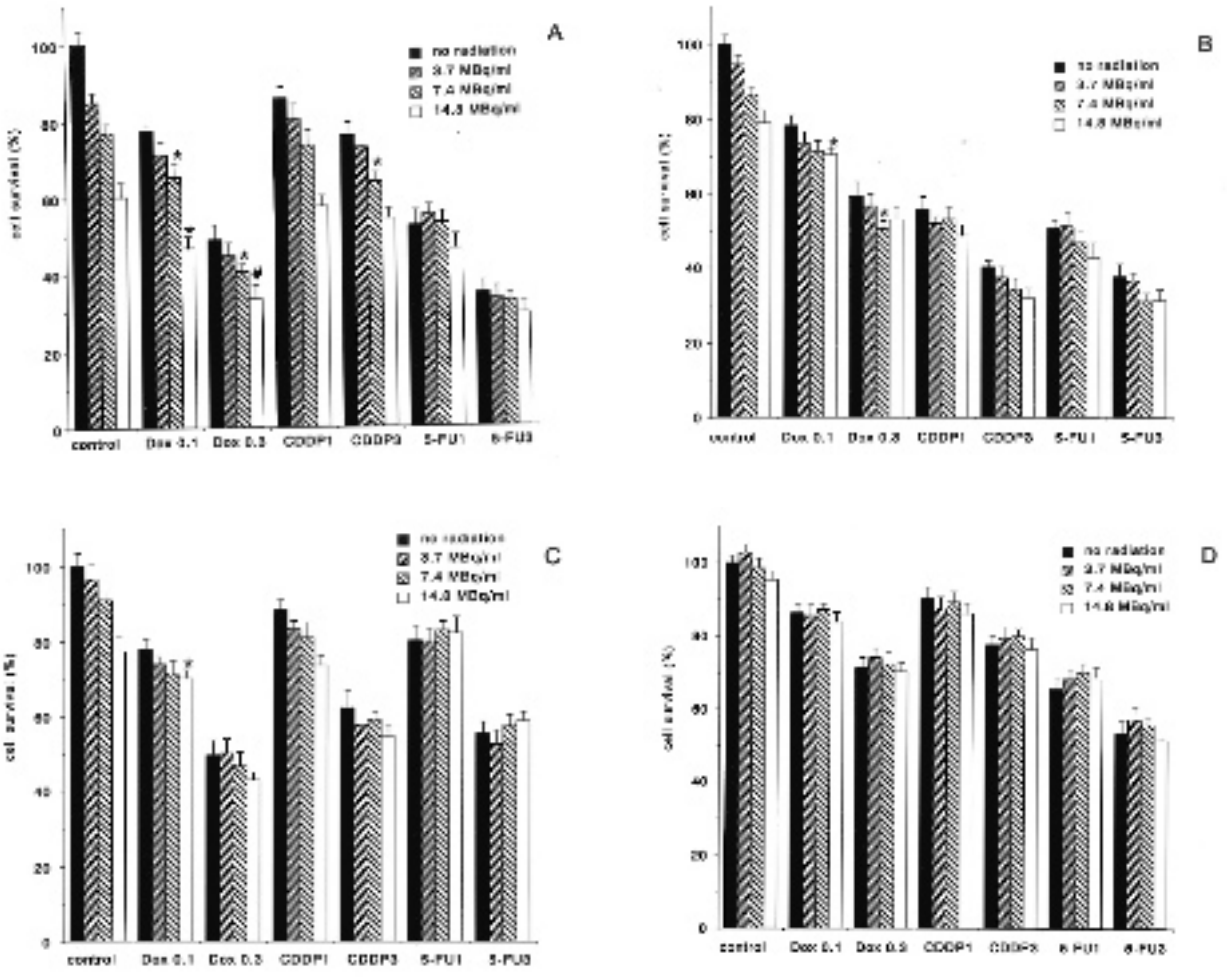

Fig. 3 Effect of I-131 irradiation combined with clinical concentrations of antineoplastic agents on survival of thyroid cancer cells. A: FRO, B: NPA, C: WRO, D: B-CPAP. CDDP = cisplatin, Dox = doxorubicin, 5-FU = 5-fluorouracil; numbers following these abbreviations signify experimental concentrations of the drug in μg/m*l*. Bars represent standard deviation of triplicate samples. * indicates combinations which showed significant difference ($p < 0.05$) both from irradiation alone and from drug alone, whereas # denotes the combination with more significant ($p < 0.01$) additional effect on both.

and WRO, and caused only minimal change in B-CPAP. When used together with clinically achievable doses of chemotherapeutic agents, the combined effect was most demonstrable in FRO, followed by NPA and WRO, and not significant in B-CAP (Fig. 3A–D). And among the antineoplastic drugs added to radioiodine, Dox, CDDP and 5-FU had substantial, marginal, and nonsignificant effects, respectively. In FRO cells, chemo-radionuclide therapy with 14.8 MBq of I-131 and half-lethal doses of Dox (0.3 μg/m*l*), CDDP (3 μg/m*l*), and 5-FU (3 μg/m*l*) reduced cell survival to 29.4, 54.9, and 33.4% of the control vs. 60.2% with radiation alone. Of these 3 combinations, Dox-radioiodine was significantly more cytotoxic than the drug alone ($p < 0.01$) and than radiation alone ($p < 0.01$), whereas I-131 failed to produce any significant additional effect on CDDP or 5-FU.

DISCUSSION

Thyroid cancer has usually been thought to be relatively resistant to antineoplastic agents, due presumably to its slow rate of proliferation, but some authors have reported using chemotherapy for inoperable and/or anaplastic tumors, with Dox being the most widely used drug.[15,16] Dox was also employed in combination with external beam radiotherapy for locally advanced thyroid carcinoma, with high rates of initial local tumor control both in well-differentiated and anaplastic types.[17] Surprisingly, however, we could not find any articles in the literature regarding simultaneous use of RAI and Dox or other tumoricidal agents, despite there being a multitude of experimental studies on chemical enhancement of other forms of internal radiation such as radioimmuno-

therapy[18,19] and transarterial injection of radiolabled Lipiodol.[20]

Keeping future clinical applications in mind, for combination experiments with radioiodine, we used antineoplastic drugs at or near the peak serum concentrations after administration of clinically accepted amounts: the values indicated in package inserts were 1.5 μg/m*l* for CDDP, 0.3 μg/m*l* for Dox and 15 μg/m*l* for 5-FU. Among the 3 agents tried, Dox exerted significant cytotoxicity within the clinically tolerable dose, with further potentiation by RAI. These results are concordant with the good response observed in clinical tumors to the combination of Dox and external irradiation[17] as well as with the reported *in vitro* synergistic effect of I-131 and Dox on proliferation of a hepatoblastic cell line, HepG2.[20] As in HepG2, CDDP was not so effective either alone or with RAI in the thyroid cancer cell lines we used: the half-lethal doses exceeded the usual clinical concentrations. Lastly, 5-FU was not as synergistic with RAI in the thyroid cancer cells as in HepG2. Although moderately toxic when used alone, the addition of I-131, with even the highest used dose of 14.8 MBq/m*l*, resulted in only minimal, insignificant additional cytoreduction in NPA but not in other cells (Fig. 3). This discordance can be attributed to variations in sensitivity to the drug among different lineages.

The time-dependency of 5-FU is well-known.[4,21] Our present study suggests that this drug is not very hopeful as a potentiator of I-131 therapy for thyroid cancer, but if in the future we use this drug to enhance radionuclide treatment, we should consider means to ensure constant exposure during internal radiation.

Among the potential theoretical mechanisms for synergy between anticancer drugs and ionizing radiation (reviewed in Ref. 5), most pertinent to our experimental setting will be attacks on different points of cancer-cell replication. As demonstrated above, continuous irradiation with I-131 also showed beneficial enhancement in concomitant chemotherapy with Dox at least *in vitro*. Moreover, while it is obvious that our initial experimental results will not automatically translate into successful clinical applications in the near future, chemo-radiotherapy has additional benefits *in vivo* that are not expected in the homogeneous cell population of cancer lines; radioresistant fractions of cells or lesions may still be sensitive to antineoplastic agents.[5,7] Therefore, it could be expected that if used clinically in the future, Dox could attack thyroid cancer cells within macronodular lesions which had insufficient and/or inhomogeneous uptake of RAI.

The experimental protocol of overlaying radioactive culture medium on a monolayer of cancer cells simulates the ideal condition *in vivo* of 100% retention of RAI. In that setting, we estimate the absorbed dose of radiation to cancer cells[22] as follows: in plastic multiwell plates, it can be assumed that distribution of the radionuclide is uniform and that only non-penetrating radiation within the medium in a well delivers a significant dose, whereas the contribution of penetrating gamma rays is negligible: by using published data from ICRP,[23] our calculation found that cells incubated for 48 hr with 3.7–14.8 MBq/m*l* of I-131 in the medium theoretically absorb about 18–72 Gy.

Before widely applying our favorable research data to clinical practice, we have to clarify many biological uncertainties, such as the difference between monolayer culture and spherical, three-dimensional tumors,[24] the difference between differentiated *in vivo* cancer cells and established, fast growing cell lines, changes in kinetics and distribution of radionuclide by chemotherapy, and increased morbidity due to the combination. Furthermore, the logistics of drug delivery also needs to be optimized regarding the route of administration (althogh Dox and CDDP are for infusion only, 5-FU or its derivatives can be given orally), the possibility of multidrug use, and the time schedule. Since preliminary evaluation *in vitro* had similar effects of the chemo-radioiodine combination regardless of which was applied first (data not shown), it may work better *in vivo* when RAI is given prior to antineoplastic drugs to avoid reduction in uptake of the radionuclide due to cell damage caused by chemotherapy.

In conclusion, our initial data warrant future animal studies and feasibility trials in a limited number of patients to achieve more accurate characterization and further refinements.

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