In Vitro Demonstration of Synergy Between Radionuclide and Chemotherapy

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Radionuclide therapy is currently used in the treatment of some malignancies, including hepatocellular carcinoma. The effects of external beam radiotherapy are improved by combining it with chemotherapy. The aim of this study was to determine whether such a synergistic effect could be demonstrated in vitro with internal radiation therapy. **Methods:** HepG2 cells were cultured from Day 0 to Day 8 under the following conditions: exposure for 4 hr on Day 2 to increasing concentrations of 5-fluorouracil (5FU), doxorubicin or cisplatin (CDDP); exposure from Day 2 to Day 8 to increasing concentrations of ^{131}-Iiodide; exposure on Day 2 to low-toxicity doses of drugs for 4 hr, followed by exposure to ^{131}I at increasing concentrations; and exposure to increasing concentrations of ^{131}I from Day 2 to Day 8, with exposure for 4 hr on Day 6 to the drugs. Cell toxicity was assessed by enzyme release (lactate dehydrogenase and aspartate aminotransferase) in the culture medium and on cell survival (protein and tetrazolium dye test). All cultures were run in triplicate. **Results:** A dose- and time-dependent toxicity was demonstrated with doxorubicin and CDDP but not with 5FU. When HepG2 cells were exposed to ^{131}I, the toxicity was rather low, but significant, and was time- and dose-dependent. Treating these cells with combination radiotherapy and chemotherapy resulted in a toxicity that was significantly greater than that with ^{131}I or chemotherapy drugs alone. **Conclusion:** The radiosensitivity of HepG2 cells is low; combining a chemotherapeutic drug with a radiotherapeutic agent improves the radiosensitivity in a synergistic fashion. This combination is thus able to strengthen the therapeutic effect of internal radiation therapy in different malignancies, particularly in hepatocellular carcinoma.

Key Words: radionuclide therapy; iodine-131; chemoradiotherapy

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Internal radiation therapy with nonsealed sources has some indications in cancer therapy. Newly developed compounds allow precise tumor targeting and, when coupled with appropriate radionuclides, provide a means of effective treatment for certain types of cancer. Iodine-131-labeled metaiodobenzylguanidine (MIBG), radiolabeled monoclonal antibodies and radiolabeled somatostatin have opened new avenues for treatment. Arterial embolization techniques can also be used to selectively deliver the therapeutic agent directly at the tumor level. For primary or secondary liver tumors, glass microspheres (*1–3*) and Lipiodol are especially useful as vectors. We have developed the ^{131}I-labeled Lipiodol method (*4–8*) and have obtained promising therapeutic results in hepatocellular carcinoma. Evidence of a therapeutic advantage for the concomitant combination of external radiotherapy and chemotherapy has been obtained with different tissue and cell models (*9,10*) and in various animal tumors (*11*). These combinations are now widely used for several types of malignant tumors (*12*). Their development is one of the main avenues of clinical research in oncology. It would also be important to know whether the effect also occurs when irradiation is delivered, not as brief fractions of external irradiation but as a continuous low dose, as is the case with nonsealed source internal radiation therapy. We, therefore, conducted a study using a liver cell tumor model to determine whether combining radiotherapy agent ^{131}I and a chemotherapy agent could provide a therapeutic advantage.

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MATERIALS AND METHODS

Cell Cultures

A human hepatoblastoma cell line, HepG2, obtained by Knowles et al. (*13*) was maintained in a basic medium containing 75% minimum essential medium and 25% 199 medium supplemented with 10% fetal calf serum.

Cells were seeded on multiwell culture plates containing the above medium and 7×10^{-7} *M* hydrocortisone hemisuccinate but not fetal calf serum. Cells were then incubated at 37°C in a humid atmosphere containing 95% air and 5% CO_2.

Drugs

The following anticancer drugs were used: 5-fluorouracil (5FU) (Laboratoires Roche, Neuilly, France), cisplatin (CDDP) (Laboratoires Roger Bellon, Neuilly sur Seine, France) and doxorubicin (Pharmacia, Saint-Quentin-Yvelines, France). The stock solutions of these three drugs were prepared under sterile conditions by dilution in a 5% dextrose solution. For final use, the stock solutions were diluted in the culture medium.

Radioactive Iodine

Radioactive iodine was ^{131}I (CisBiointernational, Gif sur Yvette, France), provided as $Na^{131}I$. The specific activity was 100 mCi/ml (5000 Ci/g iodine), and the radionuclidic purity was >99%.

Assessment of Cell Toxicity

Enzyme Assays. Extracellular lactate dehydrogenase and/or aspartate aminotransferase activities were measured in the culture medium to determine cytotoxicity scores with standard lactate dehydrogenase (Laboratoires Roche) and aspartate aminotransferase (Boehringer Mannheim, Meylam, France) kits using a Cobas-Bio autoanalyzer. Results were expressed per mg of protein.

Total Protein. Cells were washed then sonicated in 1 ml of phosphate-buffered saline (PBS). Protein content was measured in the cell lysate according to the Bradford method (*14*). A standard curve was obtained using a 700 μg/ml stock solution of bovine serum albumin.

Assessment of Cell Survival

Cell survival was determined either from the protein content in the cell sediment or using the tetrazolium dye (MTT) test (*15*), which measures mitochondrial succinate dehydrogenase activity in live cells. After the addition of MTT (Sigma Chemical Co., St. Louis, MO), the plates were covered and reincubated at 37°C for 2 hr, the optimal time for formation of formazan salts. The supernate was then extracted, formazan was dissolved in dimethylsulfoxide (Farmitalia carbo Erba, Paris, France) and absorbance was determined at 540 nm.

Toxic Effect of the Chemotherapy Agents

The following concentrations of drugs were studied: 5FU, 1–1000 μg/ml; CDDP, 0.1–20 μg/ml; and doxorubicin, 0.1–20 μg/ml. All tests were run with a series of control cultures.

The cells were seeded on Day 0. On Day 2, the drugs were added to the culture at the test concentrations for 4 hr. The medium was then removed, the cells were rinsed with PBS and fresh culture medium was added. This medium was renewed every day through Day 6. Medium and cell sediment were collected daily for assay.

Toxic Effect of Iodine-131-Iodide

Doses of ^{131}I-iodide doses ranged from 10 to 200 μCi/ml. Cells were seeded on Day 0. At Day 2, radioactive sodium iodide at the test concentration was added to the culture medium. This culture medium was recovered daily from Day 2 to Day 8 and replaced with fresh medium containing radioactive sodium iodide. Medium and cell sediment were collected every 2 days for assay.

The absorbed dose was calculated using a simulation program, assuming that the radioactivity was homogeneously distributed in the medium.

Combination of Chemotherapy with Iodine-131-Iodide

Cultures were seeded on Day 0. On Day 2, they were pretreated with the chemotherapeutic agent for 4 hr. The concentration of the agent, as determined from preliminary runs on drug toxicity, were such that mean cell death was in the 30%–50% range. After washing with PBS, the cells were then incubated from Day 2 to Day 8 in culture medium containing the test concentrations of ^{131}I.

Cell survival was simultaneously determined for the four test conditions:

1. No therapeutic agent;
2. Chemotherapy agent alone at the defined concentration;
3. Iodine-131-iodide alone at the different test concentrations; and
4. Combination chemotherapy (defined concentration) with ^{131}I-iodide at increasing concentrations.

Influence of Order of Administration

The test conditions were the same as described above except for the order of administration. In a simultaneous supplementary run, cells were cultured from Day 2 to Day 8 in presence of radioactive medium, and the chemotherapy agent was added on Day 6 for 4 hr.

Statistical Analysis

All experiments were performed in triplicate. Results were expressed as mean ± s.e.m. Student's t-test was used for intergroup comparisons with a significance threshold set at $p < 0.05$.

RESULTS

Sensitivity of HepG2 Cells to Cisplatin and Doxorubicin But Not 5-Fluorouracil

A major toxic effect was observed with CDDP and doxorubicin, with a significant dose-dependent 15-fold increase in enzyme release observed beginning on Day 3. This effect was exhibited for doxorubicin concentrations of ≥0.1 μg/ml and for CDDP concentrations of ≥1 μg/ml. The effect increased from Day 3 to Day 6 with CDDP. A toxic effect was observed with 5FU starting on Day 3, but it was less pronounced (4-fold factor) and did not persist. Furthermore, no dose dependence was observed.

Cell survival data are presented in Figure 1. With doxorubicin, cell death was noted early (Day 3), was dose-dependent, manifested at doses of ≥0.1 μg/ml and increased from Day 3 to Day 6. Similar results were seen with CDDP: early cell death (Day 3), dose dependence at concentrations of ≥1 μg/ml and increasing effect from Day 3 to Day 6. The effect of 5FU on cell survival was less pronounced: increased cell death was not observed on Day 3 except for high doses (1000 mg/ml), was not dose-dependent and did not increase from Day 3 to Day 6.

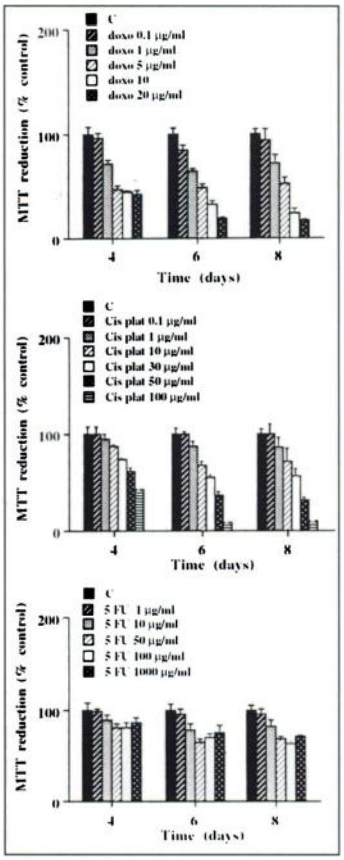

FIGURE 1. Effect of doxorubicin (doxo, upper), CDDP (Cis plat, Middle) and 5FU (lower) at increasing concentrations on HepG2 cell survival as determined by the MTT test. HepG2 cells were incubated for 4 hr on Day 2 with the chemotherapeutic agent. Assays were repeated daily. Values are expressed as mean ± s.e.m. for three tests.

The results obtained with the MTT test and protein assay were equivalent.

Sensitivity of HepG2 Cells to Iodine-131-Iodide

Cell cultures maintained in the presence of ^{131}I-iodide showed moderate cytotoxicity (2-fold factor on Day 3) that was dose dependent and occurred late (starting on Day 4 or Day 6), with no progression of the effect with duration of exposure.

Cell death (Fig. 2) was moderate and dose dependent, with little difference between Day 6 and Day 8.

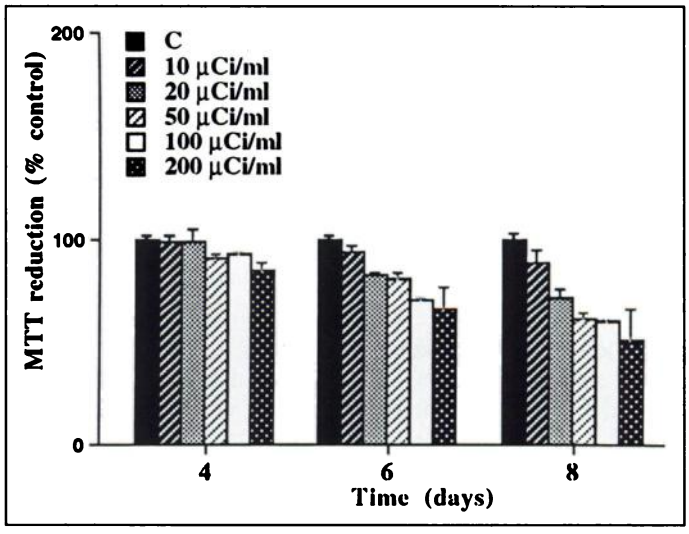

FIGURE 2. Demonstration of the efficacy of ^{131}I-iodide at increasing doses on HepG2 cell survival, as determined with the MTT test. HepG2 cells were incubated from Day 2 to Day 8 with ^{131}I-iodide. Assays were repeated daily. Values are expressed as mean ± s.e.m. for three tests.

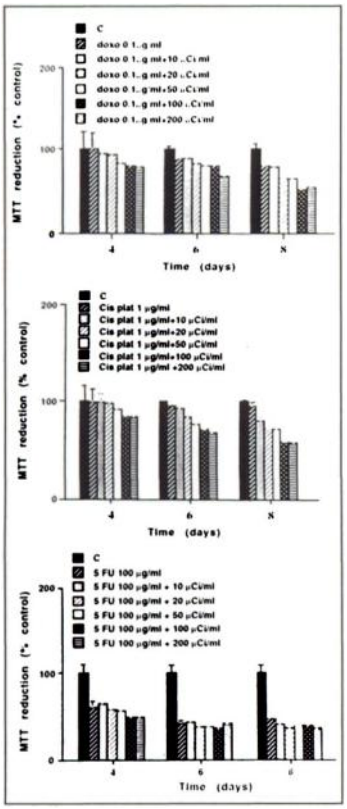

FIGURE 3. Effect of doxorubicin (doxo, upper), CDDP (Cis plat, Middle) and 5FU (lower) at one concentration in combination with increasing doses of ^{131}I-iodide on HepG2 cell survival, as determined by the MTT test. HepG2 cells were incubated for 4 hr on Day 2 with the chemotherapeutic agent, then from Day 2 to Day 8 with ^{131}I-iodide. Assays were repeated daily. Values are expressed as mean ± s.e.m. for three tests.

The radiation absorbed dose was estimated from about 4.5 Gy for a specific activity of 10 µCi/ml in the medium to 90 Gy for an activity of 200 mCi/ml.

Synergistic Action of Chemotherapy and Iodine-131-Iodide

Doxorubicin- and CDDP-induced cell death was also evident when they were used in combination with ^{131}I-iodide (Fig. 3). The addition of ^{131}I-iodide led to increased cell death, as compared with cultures with chemotherapy agents alone. This increased cell death was significant on Day 6 and Day 8 with an increase in the 25%–50% range. Increased cell death was particularly evident with the doxorubicin/^{131}I-iodide combination and with the CDDP/^{131}I-iodide combination. The toxic effect of 5FU was not significantly increased in cultures with added ^{131}I-iodide, regardless of the dose.

On Day 8, cell survival in the control cultures was greater than that in any of the other test cultures. When ^{131}I-iodide was used with a chemotherapeutic agent (Table 1), cell survival was always lower than in corresponding cultures treated with the chemotherapy agent alone or ^{131}I-iodide alone, except for the combination of 5FU/^{131}I-iodide, in which the addition of ^{131}I-iodide did not modify survival compared with 5FU alone. This increased cell death corresponded to a synergistic effect because the survival with combined therapy was significantly less than the product of survivals observed with each treatment alone.

Influence of Order of Administration

Cell survival rates on Day 8 were comparable regardless of the order of administration and for all chemotherapy agents tested.

DISCUSSION

Four types of interaction might be expected to occur between radiotherapy and chemotherapy:

1. Spatial cooperative effect, expressing the independent activity of each treatment modality (radiotherapy affecting the primary tumor in the field of irradiation and chemotherapy affecting possible metastases outside the field);
2. Independent cytotoxicity, which would allow the use of full doses for each of the two agents;
3. Protection of normal cells against irradiation by a systemic agent; and
4. Greater therapeutic activity in the field of irradiation due to the radiosensitization effect of the chemotherapy agent.

TABLE 1
Efficacy of Treatments Expressed as a Percentage of Surviving HepG2 Cells on Day 8 Compared with Control (No Treatment) and Treatment by Radiotherapy and Chemoradiotherapy

Treatment	^{131}I-iodide (µCi/ml)				
	0	10	20	50	100
[^{131}I]Iodide alone	100 (control)	84	82	72	65
5FU (100 µg/ml)	51	54†	55†	64*	54
CDDP (1 µg/ml)	65	47*†	48*†	39†	37*†
Doxorubicin (0.1 µg/ml)	60	47*†	44*†	40†	36*†

*$p < 0.05$, combinations versus chemotherapy alone.
†$p < 0.05$, combinations versus ^{131}I-iodide alone.
$p < 0.05$ for all chemotherapies alone versus control and for 50 and 100 µCi/ml doses of ^{131}I-iodide versus control, and $p < 0.05$ for all combination treatments versus control.

Because it combines the chemotherapeutic effect outside the field of irradiation with greater therapeutic efficacy within the irradiation field, this latter type of interaction has been widely studied in clinical applications of concomitant or sequential chemotherapy/radiotherapy combinations.

The effect of external irradiation (*16*) results principally from its action on genomic DNA. The DNA defects produced can involve simple rupture of the double strand, cross-links or damage to the bases or sugars. Cell repair is, however, rapid, and sublethal or potentially lethal damage may be overcome during the interval between two radiotherapy fractions. Several types of agents can inhibit recovery from radiation-induced cell damage (*16*): chemotherapy agents, inhibitors of DNA repair (hydroxyurea, 1-β-D-arabinofuranosylcytosine and so on), halogenated pyrimidines, caffeine and agents that provoke glutathione depletion. The chemotherapy agent doxorubicin has a well-recognized additive effect when it is used with radiotherapy. Platinum salts, notably CDDP, also appear to be excellent radiosensitizing agents. CDDP may inhibit repair of sublethal or potentially lethal damage. This has been demonstrated both with classical irradiation protocols and with low-dose continuous radiotherapy. A supra-additive effect has even been obtained by delivering CDDP by continuous infusion (*16*). Conversely, this additive effect may be related to the fact that certain CDDP-induced defects are still present at the time of irradiation, further adding to its efficacy. CDDP, for example, can provoke production of free radicals or induce formation of platinum–DNA complexes.

5-Fluorouracil also has a reinforcing effect on the action of ionizing irradiation, notably when it is used after the irradiation session (*9*). Others consider this effect to be more pronounced when 5FU is administered before or during irradiation (*12*).

Our objective was to determine whether the effect of prolonged low-dose irradiation delivered by a radionuclide could be enhanced by the adjunctive use of a chemotherapeutic agent. We used the human hepatoblastoma cell line HepG2 as a model to improve the treatment of hepatocellular carcinoma by means

of combined systemic chemotherapy and radiotherapy with ^{131}I-iodide. We also ran similar tests (data not shown) with three other cell lines derived from human hepatocellular carcinomas (Hep3B, HuH7 and PLCPRF) and obtained results similar to those reported here. The three drugs tested (doxorubicin, CDDP and 5FU) all have known radiosensitizing effects; all three are known to be among the most effective chemotherapy agents in hepatocellular carcinoma. Use of ^{131}I-iodide in our tests rather than radiolabeled Lipiodol eliminated any problem related to contact between the oily surface phase and deep cell layers. The parameters studied were cell survival (assessed by protein assay and the MTT test) and cell toxicity (enzyme assay). The two methods used to evaluate cell survival are widely recognized classical methods, and equivalent results were obtained with both. Other more complex techniques could have been used (Ki67 or proliferating cell nuclear antigen immunolabeling, clonogenicity test, incorporation of tritiated thymidine and so on) but would have been difficult to perform and interpret because of the presence of ^{131}I-iodide. For similar reasons, we did not study the cellular mechanisms involved.

Duration of treatment was limited to 6–8 Days, which may appear to be short considering the long half-life of ^{131}I-iodide. It was, however, difficult to prolong the study because cell confluence is reached spontaneously by about Day 4 or Day 5. Confluent cells are much more resistant to various therapeutic agents (*10*) and, consequently, the results would have been biased. The cross-irradiation from other incubation wells was considered negligible: distance between plastic wells was about 10 mm and then the cross irradiation could only be related to gamma irradiation. In the worst case (200 μCi/ml), the calculated dose related to the cross-irradiation was estimated at 36 mGy.

The first step was to evaluate the toxic effects and cell survival after chemotherapy or radiotherapy alone. We were able to confirm the efficacy of CDDP and doxorubicin and a weaker effect of 5FU. Irradiation had a lower toxicity in the model used. This might be explained by the typical radioresistance of hepatocellular carcinoma but might also result from experimental conditions because exposure to irradiation was limited in time and high doses of ^{131}I-iodide could not be used (because of risks to operator safety). The second step concerned the combined use of a chemotherapy agent and ^{131}I-iodide. The results obtained provided interesting information, as doxorubicin and CDDP were seen to produce increased cell death in cultures treated with combination chemoradiotherapy compared with the chemotherapeutic agent alone. This is a synergistic effect (*17*), the efficacy of the combination therapy being greater than the product of the single effects. Such an effect was not seen with 5FU. The order of administration of the therapeutic agents had no effect, cell survivals and toxicity being equivalent, if the chemotherapy agent was given before ^{131}I-iodide or vice versa. We did not test the daily administration of low-dose CDDP during irradiation, which has been suggested to give good results with classical external irradiation (*18*).

CONCLUSION

Our results with continuous low-dose radionuclide irradiation confirm those obtained with classical external irradiation, which have been the basis for chemoradiotherapy combinations and the subsequent progress in the treatment of several cancers. Our findings suggest the adjunction of chemotherapy could be proposed in selected patients who might be expected to benefit from nonsealed internal radiation therapy. Our study cannot be used to define the components of such a combination, but it would suggest that CDDP could be used because it is easy to handle and administer before unsealed source therapy.

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