Effects of Combined Treatments of cis-Diamminedichloroplatinum(II), 5-Fluorouracil, and X-Rays on Growth of Human Cancer Nodules Maintained in Continuous Organotypic Culture

R. Beaupain and C. Dionet

ABSTRACT

The effectiveness of combined treatments of cis-diamminedichloroplatinum(II) (cis-DDP), 5-fluorouracil (5-FUra), and X-rays on growth inhibition of human pulmonary cancer nodules maintained in continuous organotypic culture was tested. To obtain the most effective growth inhibition, a cis-DDP treatment had to be preceded by an X-irradiation, whereas a 5-FUra treatment had to be postirradiated. This indicates the importance of the order in which the different combinations must be done. A mixture of 5-FUra and cis-DDP was even more effective than the X-ray and drug combination but only for a short period of time; reduction of nodule size and cell loss occurred during the 2 weeks following the treatment. After this period, as happened also with nodules treated with 5-FUra alone, vigorous regrowth occurred after treatment with cis-DDP plus 5-FUra, and all the nodules regained the size of the controls sooner or later. It is to be noted that, if X-rays were applied after a 5-FUra treatment, this regrowth is inhibited at least up to 45 days after the treatment.

INTRODUCTION

Antitumoral agents often present undesirable side effects in patients, and attempts have been made to reduce these effects by combining several drugs, or drugs and ionizing irradiations at lower doses than generally used (1), in the hope that such combinations would potentiate the individual cytotoxic effects of the drugs and improve therapeutic results (2). It is of course important to know the optimal sequencing of cytostatic treatments. In order to study the effects of the different treatment schedules, a mouse in vivo model has been used combining cis-DDP, 5-FUra, and X-rays (3). In this case, the choice of cytotoxic agents was made according to their action on the different phases of the cell cycle. It is clearly established that cis-DDP acts mainly on the G1 phase of the cell cycle (4). 5-FUra interferes with the S phase (5), the most radiosensitive phase (6), while ionizing irradiation acts on cells in M and G2 phases (5, 7). In the mouse leukemia model (3), a combination of cis-DDP and 5-FUra cured Li210 leukemia in mice at drug levels which were ineffective when used alone, and X-rays potentiate the antitumoral activity of these 2 drugs.

In the present study, we applied combinations to human cancer cells cultivated in tridimensional organotypic culture (8), which was derived from the method of Wolff and Wolff (9). This culture system has several advantages. The 3-dimensional architecture of the cells and some cell differentiation are maintained. Moreover, the low traumatizing culture conditions make it possible to maintain injured cells in situ, without touching them, and offer them a maximal survival chance, close to the situation in vivo. It is to be noted that subculturing was done without enzymatic dissociation of the cells; nodule growth measurements were made without handling the nodules, and also that treated nodules were subcultivated by transferring them gently onto a fresh medium. It has been shown in a previous report (8) that the organotypic cancer nodules resist X-rays and drug (cis-platinum) treatment more than do the same cells cultivated as a monolayer (10). Thus, it seems interesting to use this culture method to test the effectiveness of cytostatic treatment combinations on nodule growth, survival, and regeneration.

MATERIALS AND METHODS

Organotypic Culture. The organotypic culture method has been described elsewhere (8). The A549 nodules (originally monolayer cells, coming from an alveolar II lung adenocarcinoma (11)) were maintained in continuous culture on a semisolid agar medium containing 1% Bacto-agar (Difco) in distilled water mixed in a 1:1 ratio with 2 x concentrated RPMI 1640 culture medium supplemented with 20% fetal bovine serum. The nodules were routinely subcultured every 10 days by dividing them in 2 (or more) pieces with microscissors. Then, they were placed on a fresh medium (2 ml) previously prepared in Petri dishes with a tight lid (Falcon 1006).

Drug and X-Ray Treatments of the Nodules. Experiments were done 5 days after subculturing, when the nodules have finished wound healing. The cultures were treated with X-rays (4 Gy), with cis-DDP (a gift from Laboratoires Roger Bellon; 15 μg/ml for 6 h), with 5-FUra (obtained from Products Roche) (2.5 mg/ml, 6 h), or with 2 combinations of the cytostatics. The drug treatments were done by immersing the nodules in 1.5 ml liquid RPMI 1640 containing the drug. After the treatment, the nodules were rinsed 3 times with fresh medium and were returned on the semisolid medium. For X-irradiations, a Vega machine was used working at 11 ma and 250 kV, the X-rays were filtered with 2-mm aluminum. Dose rate was 1 Gy/min, and the exposures were verified with a dosimeter (DOSIX) during the irradiation.

Measurements of Nodule Growth. In order to have an idea of nodule growth, 2-dimensional measurements were done by means of a stereomicroscope equipped with an ocular micrometer. The nodules were measured every 5 days and transferred onto a fresh medium every 10 days. The "size" (S) of the nodules was expressed in sq mm, S0 being the size of the nodule before treatment and S, the size at time t after treatment; the nodule growth was given as the percentage S/S0 for each individual nodule at the different times of measurement. For the same treatment, 5 nodules were used, and each treatment was done at least 3 times. Regression lines were calculated from the obtained values, and growth inhibition (or stimulation) was established by comparing the slopes obtained from the treated nodules with those of the controls.

1Recipient of INSERM Grant CRL 813002. To whom requests for reprints should be addressed.
2Recipient of an ARC grant.
3The abbreviations used are: cis-DDP, cis-diamminedichloroplatinum(II); 5-Fura, 5-fluorouracil.
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When the obtained growth curve was not a straight line, it was divided into 2 or more straight lines to be able to compare the slopes. Finally, in Table 1, data obtained from all the nodules used were assembled, and growth was expressed as a growth percentage per day for the controls and the differently treated nodules.

RESULTS

The results of the different series of experiments are illustrated by the data of one typical experiment in the series. The data obtained from all the treated nodules of the different series are summarized in Table 1.

Effects of Single Treatments. Chart 1 shows the growth evolution over 45 days of control nodules and of a single dose of X-rays (4 Gy), cis-DDP, and 5-FUra treatments. The control nodules had a growth rate of 6.56 ± 0.31% (SD)/day. The growth rate of nodules was 4.93 ± 0.48%/day for a treatment with 4 Gy of X-rays, 3.13 ± 0.45%/day for a treatment of 6 h with cis-DDP (15 μg/ml) and 1.25 ± 0.52%/day of a treatment of 6 h with 5-FUra (2.5 mg/ml). In other words, growth inhibition was 1.33-fold for X-rays 2.09-fold for cis-DDP, and 5.24-fold for 5-FUra compared to controls. Following treatments with either X-rays or cis-DDP, there was no resumption of nodule growth at 45 days whereas, after treatment with 5-FUra, nodule growth resumed 2 weeks after exposure. In the last case, the daily growth percentage reached that of the controls, i.e., 6.46 ± 0.76%.

Effects of an X-irradiation Preceding the Drug Treatments. When 5-FUra treatment was preceded by a 4-Gy irradiation, up to 2 weeks, the growth rate observed was in the same range as that observed after a single treatment with 5-FUra alone (Chart 2). However, for a later time of posttreatment, the growth rate was 5.03 ± 0.34%/day. The slowing down of growth compared to controls was consequently 1.30 times and the combined treatment was 1.2 times less effective than was the sum of separate treatments. A far greater effectiveness was obtained when cis-DDP was used in combination with X-rays (Chart 3). Growth rate was 0.89 ± 0.34%/day, the inhibition was consequently 7.37 times more than in controls. The sum of the inhibition effects of the separate treatments was only 3.42-fold. The X-rays potentiated the action of cis-DDP, and the effect of the combination was highly synergistic.

Effects of a Postirradiation. No synergistic effect was found when cis-DDP-treated nodules were subsequently irradiated (Chart 4). The daily growth percentage of the nodules was 2.44 ± 0.14%; compared with the controls, inhibition was 2.69 times greater (2.09 times for the nodules treated with cis-DDP alone; the sum of the growth inhibition rates of the separate treatments resulted in a 3.42-fold inhibition).

Postirradiation of 5-FUra-treated nodules was highly efficient (Chart 5). Growth rate was 1.16 ± 0.04%/day during the entire period of the experiment. The vigorous regrowth which was observed after a single 5-FUra treatment did not occur in this case. The nodules grew 4.80 times more slowly after the combined treatment than did the controls. The sum of the growth delays of the separate treatments was 3.42, and here again a synergistic effect was found.

Effects of a Mixture of cis-DDP and 5-FUra. Reduction of nodule size was observed in all the treated nodules (Chart 6), but 2 weeks after exposure to the drug mixture some regrowth occurred (growth rate, 3.33 ± 0.07%/day), and this process accelerated, attaining a growth rate of 12.25 ± 0.6%/day (1.87-fold acceleration when compared with the controls). All the treated nodules finally reached the size of the control nodules with a broad spectrum in the kinetics of resumption of growth; the fastest time for this was 57 days (Chart 7), and the slowest was 83 days.

Table 1

Comparison of growth percentage per day of A549 nodules after the different cytostatic treatments

<table>
<thead>
<tr>
<th>Time (days) after treatment</th>
<th>Growth %/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.56 ± 0.31%</td>
</tr>
<tr>
<td>4 Gy</td>
<td>4.93 ± 0.48%</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>3.13 ± 0.45%</td>
</tr>
<tr>
<td>5-FUra</td>
<td>1.25 ± 0.52%</td>
</tr>
<tr>
<td>15-45</td>
<td>6.46 ± 0.76%</td>
</tr>
<tr>
<td>cis-DDP + 5-FUra</td>
<td>3.33 ± 0.07%</td>
</tr>
<tr>
<td>30-45</td>
<td>12.25 ± 0.6%</td>
</tr>
<tr>
<td>4 Gy + cis-DDP</td>
<td>2.46 ± 0.37%</td>
</tr>
<tr>
<td>25-45</td>
<td>0.89 ± 0.34%</td>
</tr>
<tr>
<td>cis-DDP + 4 Gy</td>
<td>2.44 ± 0.14%</td>
</tr>
<tr>
<td>4 Gy + 5-FUra</td>
<td>1.77 ± 0.16%</td>
</tr>
<tr>
<td>15-45</td>
<td>5.03 ± 0.34%</td>
</tr>
<tr>
<td>5-FUra + 4 Gy</td>
<td>1.16 ± 0.04%</td>
</tr>
</tbody>
</table>

*Mean ± SD of all treated nodules.*
DISCUSSION

The aim of using 3-dimensional culture methods is to maintain in vitro the architecture and the properties of tumor cells in vivo. Recent studies have stressed the influence of the 3-dimensional state on cell functions. Thus, DNA and RNA synthesis are affected by cell shape (12), and dedifferentiated chondrocytes reexpress the differentiated phenotype when cultivated in a spherical configuration in gel culture (13). Melanoma cells cultivated as spheroids have an increased ability to form metastasis compared with the same cells cultivated as a monolayer (14). The growing interest in 3-dimensional cultures has been demonstrated recently in reports of methods using, for example, calcium alginate capsules (15) or chick embryonic tissue (16) as culture substrates. All these facts emphasize the interest of culture models which conserve as closely as possible the cellular structures of the in vivo situation while keeping cell trauma to a minimum. In the organotypic culture model, growth inhibition, survival percentage of the nodules, and the growth rhythm of regenerated nodules can be studied after the different antitumoral treatments. Although on organotypic nodules it is difficult, when using 2-dimensional growth measurements, to distinguish delay in cell division or prolongation of the cell cycle versus cell killing, a long-term slowing down (or acceleration) in the growth of healthy nodules may indicate a prolongation (or acceleration) of the cell cycle. It is very probable that the cell cycle varies according to the location of the cells in the nodules as is the case in solid tumors (in vivo (5)). We observed, however, that a cis-DDP treatment in A549 monolayer cells occasioned a significant long-term increase in the cell cycle (10). When cell killing occurs, the nodules lose their differentiation patterns (alveoli), and they tend to disintegrate. If these nodules are left on semi-solid medium without touching during the medium changes (8),...
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Chart 5. Effects of a 5-FUra treatment followed by a 4-Gy irradiation. Evolution of growth of the untreated controls (●), after a 4-Gy X-irradiation (▲), after a 5-FUra treatment (■), and after a 6-h 5-FUra treatment followed immediately by a 4-Gy irradiation (★).

Chart 6. Effects of combined cis-DDP and 5-FUra treatment. Evolution of nodule growth of the untreated controls (●), after 5-FUra treatment alone (■), cis-DDP treatment alone (▲), and a 6-h treatment with cis-DDP plus 5-FUra (★).

Chart 7. Evolution of nodule size (in sq mm) of untreated nodules (●) and of a nodule treated with cis-DDP plus 5-FUra (▲).

viable cells will have a maximal surviving chance of developing regeneration clusters in the necrotic tissue. This "cloning in situ" method permits a higher survival rate than in the case of nodule transfers. In an earlier paper (8), we reported 96% (43 of 45 treated nodules) survival of A549 nodules treated with doses of cis-DDP as high as 300 µg/ml (a 1-h treatment) using in situ cloning, while no survival was observed when the nodules were simply gently transferred (8) or dissociated and cloned. Cell survival of dissociated nodules after a 1-h treatment with cis-DDP (200 µg/ml) was 0.00089%.4 These observations stress the role even slight cell traumas play in survival, and they suggest the similarity of the role of the autofeeder cells (16) and the nonviable cells in treated nodules which offer viable cells maximum feeding conditions.

In our study, the single doses of X-rays and cis-DDP produced growth inhibitions of 25 and 52%, respectively, and we wanted to see to what degree combinations would improve the results. 5-FUra alone was apparently more effective but only during the 15 days following treatment, and we wanted to know if the observed regrowth could be avoided.

Present data show that preirradiation of cis-DDP treated nodules was more effective than was postirradiation. A possible explanation is that X-rays slow down the cell cycle (17) resulting in a potentiation of the cis-DDP treatment which acts better on cells with a slowed down cell cycle (18-20). In Chinese hamster ovary cells, an X-ray dose-dependent delay was observed during the G1 phase (7), the most cis-DDP-sensitive phase (4). It should be noted that in the L1210 mouse leukemia model the optimal efficiency of X-ray and cis-DDP combination was obtained with the inverse sequencing (3), but in a solid tumor model in vivo (mouse sarcoma)5 the most efficient sequencing was the same as for the organotypic nodules. Likewise, A549 nodules treated with misonidazole showed growth stimulation as was the case in a rat chondrogenic sarcoma, while A549 cells treated as a cell suspension did not (21). These 2 examples may support the interest of the organotypic culture model and suggest that survival may be quite different for isolated cells and for solid tumor cells. These data emphasize also how important it is to compare the different schedules for applying drugs and X-rays, on monolayer cultures, on 3-dimensional cultures, and on in vivo tumors and to study their efficiency for each individual type of cancer. As a matter of fact, a radiosensitizing effect of cis-DDP has been observed in a mouse mammary tumor in vivo (and also in lung fibroblasts in vitro) (22, 23) but not in the A549 pulmonary cancer nodules. The differences in the tumor type treated, but also the duration of treatment of the cells with cis-DDP before irradiation,

4R. Beaupain, unpublished data.

5C. Dionet, unpublished results.
may explain these opposite findings.

Data obtained with 5-FUra have shown a temporary growth arrest of the treated nodules followed by a vigorous regrowth. It is known that 5-FUra is metabolized in the cells and transformed into fluoro-5-deoxyuridine, which inhibits DNA synthesis for several days, but tissue concentrations of this 5-FUra metabolite decline and DNA synthesis recovers (24). The regrowth of the nodules 2 weeks after the 5-FUra treatment may be explained by this process.

Suppression of regrowth was actually obtained by postirradiation of the 5-FUra treated nodules. This result is in agreement with established findings that 5-FUra potentiates the effects of X-rays (5). As 5-FUra interferes with repair (5), the effect of the postirradiation may be enhanced by unrepaired X-ray damage. This idea is supported by the fact that the 5-FUra plus 4-Gy-treated nodules grew about 8 times slower than did the X-ray-irradiated nodules during the first 2 weeks after the treatment (increases of node size of about 9 and 76%, respectively) and that, in this period of time, the nodules grew 2 times slower after the combined treatment than after a single 5-FUra treatment (9 and 19%, respectively). It is possible that X-rays may occasion a greater retention of 5-FUra metabolites (24) in the nodules, and these effects may suppress their regrowth.

Cell death and cell loss occurred in cis-DDP plus 5-FUra-treated nodules, but here regrowth was not suppressed. The combined action of the drugs (cis-DDP-DNA cross-links and hindering of repair by 5-FUra) may result in cell necrosis and reduction of node size. The consecutive regrowth may coincide with the elimination of the 5-FUra metabolites, and the repair of cis-DDP-DNA cross-links (4, 25, 26) may then resume without being hindered.

In the case of the combined 5-FUra-plus-cis-DDP treatments, the node regrowth became faster than that of the controls, and all of them regained the size of the controls sooner or later. It is difficult to explain this phenomenon in the light of already known facts. Hypothetically, one may imagine selection of the fastest growing cells or modifications in the cell genome. These modifications may be due to misrepair of cis-DDP-DNA cross-links considering the mutagenic effects of cis-DDP in Chinese hamster V79 cells (27). We are now studying modifications in X-ray and drug sensitivity of cis-DDP- and 5-FUra-treated A549 nodules established as continuous lines. Accelerated growth of recurring tumors does occur in patients, and the organotypic culture method may be a tool in the study of the formation of regenerating cancers and the effects of incomplete killing of tumors.

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