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Importance of the Irradiation Timing Within a Chemoradiotherapy Sequence Including Cisplatin and 5-FU-Folinic acid. Experimental Results

J.L. Lagrange, J.L. Fischel, S. Galliani, P. Formento, T. Guillot, M. Bardon and G. Milano

The objective of the present *in vitro* study was to determine an optimal timing of the irradiation in the combination cisplatin (CDDP) and 5-fluorouracil–folinic-acid (5-FU–FA) allowing a maximal cytotoxic effect on a human cell line derived from a head and neck carcinoma (CAL 27 cells). The various tested chemoradiotherapy sequences were applied in parallel to human keratinocytes in culture (SVK 14 cells). This was done in order to define the best sequence allowing the achievement of an optimal selectivity of the cytotoxic effects. The drug sequence was: CDDP over 2 h then fresh medium was added including the tandem 5-FU–d,l FA applied 6 h after CDDP, for 5 days. Irradiation was applied only once and at various times within the drug sequence. The cytotoxicity effects of the different chemoradiotherapy combinations were assessed by the MTT semi-automated test. The part taken by the 5-FU–FA combinations in the overall cytotoxicity was examined; an effect was apparent on CAL 27 cells only. The evolution of the radiation effect (RE = cell survival after drugs/cell survival after drugs plus irradiation) was analysed as a function of the different times of irradiation within the given drug sequence. Clearly, the RE values were dependent upon time at which the radiation dose in the chemoradiotherapy regimen was administered. For CAL 27 cells, irradiation effects were maximal at the first irradiation time tested after the end of the CDDP exposure (i.e. t = 3.5 h). In contrast, this optimal chemoradiotherapy timing for better cytotoxicity on CAL 27 cells did not correspond to that of SVK 14 cells. Consequently, it was possible to establish that the best time for the selectivity index was located shortly after the CDDP exposure.
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INTRODUCTION

THE MAIN objective of concomitant chemoradiotherapy is to increase complete remission and cure rate of solid tumours [1]. An additional purpose is to reduce treatment morbidity and to increase quality of life by decreasing the incidence of standard surgical procedures, the aggressiveness of surgery, or the total radiotherapy/chemotherapy dose delivered. These objectives apply quite well to head and neck cancer where concomitant chemoradiotherapy can find a large area of application. Chemotherapy, as judged by its current optimal results obtained with the 5-fluorouracil (5-FU)–cisplatin association, reaches 80% of objective response; however complete responses represent only half of the response rate [2–4]. Due to positive interactions which have been demonstrated between 5-FU and radiotherapy

on the one hand [5] and between cisplatin (CDDP) and radiotherapy on the other [6], the concomitant use of 5-FU–CDDP and a radiation dose has been reported by many authors [7–9]. In addition, because of the enhanced cytotoxicity of 5-FU by folinic acid (FA) [10], the combination of radiotherapy and CDDP, 5-FU–FA has been tested in advanced head and neck cancer patients and has shown interesting antitumour effects associated however, with marked host toxicity including cutaneous toxicity [11]. These clinical protocols associating radiotherapy with chemotherapy including CDDP and 5-FU were highly variable for the sequence used between irradiation and administration of drugs. It was the main objective of the present *in vitro* study to search for an optimal timing of the irradiation in the combination CDDP and 5-FU–FA allowing a maximal cytotoxic effect on a human cell line derived from a head and neck carcinoma. In addition, the various tested sequences were applied in parallel to human keratinocytes in culture. This was done in order to define the best sequence allowing the achievement of an optimal selectivity of the cytotoxic effects.

MATERIALS AND METHODS

Chemicals

5-FU was obtained from Roche Laboratories (Neuilly, France) in an injectable form dissolved in water (final concentration 0.385 mol/l). CDDP was obtained from R. Bellon Laboratories (Paris, France) in an injectable form dissolved in 0.9% NaCl (final concentration 1.66 10^{-3} mol/l). FA (d,l) was obtained from Sigma (La Verpillière, France), as a powder that was dissolved just before use in water at a final concentration of 10^{-2} mol/l. These stock solutions were stored at −20°C.

The MTT test [12] was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and dimethylsulphoxide (DMSO), both from Sigma. Dulbecco's modified Eagles medium (DMEM), glutamine and fetal bovine serum (FBS) were purchased from GIBCO (Paisley, U.K.). Penicillin and streptomycin were obtained from Merieux (Lyon, France).

The CAL 27 cell line was established in our institute [13] from a squamous cell carcinoma of the head and neck. It was obtained from a tumour fragment excised prior to any treatment. Its doubling time, 35 h, was measured during exponential growth. SVK 14 cells (doubling time of 42 h) were derived from human keratinocytes and were sent to us by Dr T. Purkis (ICRF, Skin Tumour Laboratory, London).

Experimental conditions

Cells were routinely cultured in a humidified incubator (Sanyo) at 37°C with an atmosphere containing 8% CO_2 in air. Initial cell densities were 2500 cells per well (96-well plates). Cells were in the logarithmic phase of growth. The optimal sequence between CDDP, 5-FU–FA and their concentration ranges were determined from a recent *in vitro* study on CAL 27 cells [14]. In a preliminary step, dose–response curves for CAL 27 cells and SVK 14 cells were obtained. This allowed the determination of the IC_{50} values (drug concentration inhibiting 50% of cellular proliferation as compared with controls) for 5-FU and CDDP. For the tested combinations with drugs and irradiation, the highest drug concentration was close to the IC_{50} value. On this basis, the drug sequence was: CDDP (0, 1, 2, 3 μg/ml for CAL 27 and 0, 2.3, 4.6, 6.9 μg/ml for SVK 14 cells) over 2 h then fresh medium was added including the tandem 5-FU (0, 2.5, 5, 10 ng/ml for CAL 27 and 0, 25, 50, 100 ng/ml for SVK 14 cells)—FA (0, 10^{-5} mol/l) applied 6 h after the end of CDDP and for 5 days. At this time fresh medium without drugs was applied for 2 days prior to the MTT test. This resulted in 32 drug combinations being tested (four concentrations for CDDP, four concentrations for 5-FU with/without FA 10^{-5} mol/l). Irradiation was applied only once and at various times within the drug sequence: 30 min before starting CDDP, 1.5 h after the start of CDDP, 1.5, 4, 5.5, after the end of CDDP exposure (i.e. 3.5, 6, 7.5 h after the start of treatment) and 2 h, 4.5 h, 6.5 h after the start of 5-FU (i.e. 10, 12.5, 14.5 h after start of treatment). In total, 512 experimental conditions were tested for each cell line (32 drug combinations, 8 different times for irradiation/controls). According to the differences in radiosensitivity between CAL 27 cells and SVK 14 cells (see results section) the delivered dose was 2 Gy for CAL 27 cells and 3.5 Gy for SVK 14 cells. Irradiation consisted in a single exposure to γ-rays delivered by a cobalt 60 source (Teratron 780, AECL) with a rate of 1.5 Gy/min. In practice, 96-well plates were thermostated during the time of their transport (5–10 min) from the laboratory to the irradiation ward.

Evaluation of cytotoxicity

The cytotoxic effects of the different chemoradiotherapy combinations were assessed by the MTT semi-automated test [12] after 7 days of growth in 96-well incubating plates. The MTT incubation time was 4 h. Results were expressed as the relative percentage of absorbance compared to controls. Absorbance was set at 540 nm and measured on a Titerteck Twinreader. Each experimental point was performed in quadruplicate. The radiation effect (RE) was defined as the ratio between cell survival after drugs and cell survival after drugs plus irradiation. The evaluation of the MTT test for the assessment of radiosensitivity has been reported by Carmichael *et al.* [12]: the MTT test gave reproducible and comparable results to the clonogenic assay. The selectivity index was defined as

$$SI = \frac{RE\ CAL\ 27}{RE\ SVK\ 14}.$$

RESULTS

Figure 1 shows the radiosensitivity curves for CAL 27 cells and SVK 14 cells, respectively. For both cell lines, a dose–effect relationship was apparent with radiation having a more pronounced action on tumour cells than on keratinocytes. The individual effect of CDDP within the overall cytotoxicity resulting from all tested experimental conditions is shown in Fig. 2. Both CAL 27 cells and SVK 14 cells responded to the increasing doses of CDDP. In the same way, Fig. 3 examines the contribution of the 5-FU–FA combinations in the overall cytotoxicity. An effect was apparent for CAL 27 cells only where survivals were markedly reduced with the highest dose of the 5-FU–FA association. The part played by irradiation within the overall cytotoxicity is shown in Fig. 4. It is shown that for both cell lines irradiation had a specific cytotoxic effect.

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Part of these results were presented at the IV International I.S.T. Symposium on Interaction of Chemotherapy and Radiotherapy in Solid Tumors (Genoa, Italy, 13–15 April 1992)
Revised 15 Feb. 1993; accepted 19 Feb. 1993.

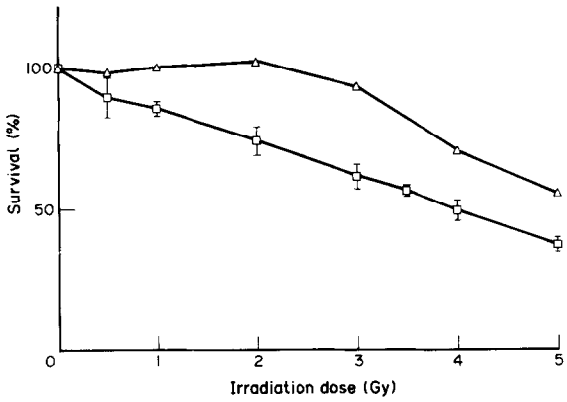

Fig. 1. Cytotoxicity as a function of the irradiation dose. Open squares = CAL 27 cells; open triangles = SVK 14 cells. Vertical bars indicate standard deviation (quadruplicates).

Figure 5 examines the evolution of the RE as a function of the different times of irradiation within the given drug sequence. Clearly, the RE values are dependent upon the time at which the radiation dose in the chemoradiotherapy regimen was administered. For CAL 27 cells, irradiation effects were maximal at the first irradiation time tested after the end of the CDDP exposure (i.e. t = 3.5 h in Fig. 5). In contrast, this optimal chemoradiotherapy timing for greater cytotoxicity on CAL 27 cells did not correspond with that of SVK 14 cells. Consequently, it was possible to establish that the best time for the selectivity index was located shortly after the CDDP exposure.

DISCUSSION

The main objective was to compare different chemoradiotherapy sequences varying the time of irradiation application. It must be emphasised that the present experiments were not specifically designed to elucidate the mechanisms of drug–radiation interactions. A CDDP–5-FU sequence which was previously validated both experimentally [14] and clinically [4] was kept constant. From a recent literature review [5] it appears that most studies indicate that irradiation followed by 5-FU is significantly more effective than the inverse sequence both *in vivo* and *in vitro*. In addition, the duration of 5-FU exposure (at least one doubling time) and the time interval before 5-FU (8–9 h) have been reported as being important [15]. Although an optimal timing between CDDP and irradiation has still to be defined [16, 17], most experiments in which CDDP exposure was applied prior to radiation resulted in enhancement of radiation response both *in vitro* and *in vivo* [5]. Our results strongly emphasise the time dependence of irradiation within a CDDP–5-FU sequence. This is, in our opinion, the main finding of the present study. An optimal time for the greatest antitumour effects was located between the end of the CDDP exposure and before the beginning of the 5-FU–FA prolonged application. This finding agrees very well with the aforementioned literature data for CDDP– and 5-FU– radiation interactions. We found 1.5 h after CDDP exposure to be an optimal time for maximal effect on tumour cells. It is quite conceivable that shortly after the end of the CDDP exposure could arguably be an even better timing since a maximal intracellular CDDP concentration could be expected. This hypothesis is based on recent experimental data showing that Pt accumulates progressively within the tumoral cells and leaves the intracellular compartment without lag time following a two-slope kinetic pattern with an α half-life of 1.29 h [18]. In addition, it was shown that the optimal irradiation time generating the best cytotoxic activity on tumour cells corresponded to a phase at which irradiation induced minimal cellular damage on keratinocytes as compared to other sequences. Thus, this optimal time was also the one which gave the best selectivity index (Fig. 5). Extrapolation to the clinical context must however be qualified by the fact that SVK 14 cells are not freshly cultured human keratinocytes; it would be interesting to confirm this aspect of an optimal selectivity index by a comparable study on an animal model.

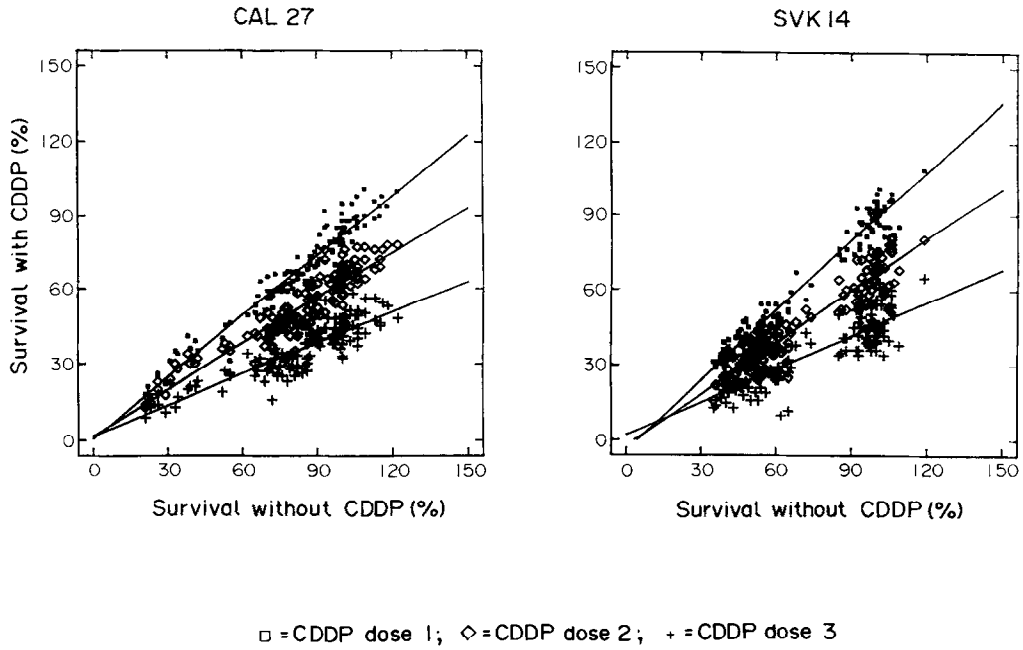

Fig. 2. Specific effect of CDDP within the total cytotoxicity resulting from all experimental conditions tested. CDDP dose 1 was 1 μg/ml for CAL 27 and 2.3 μg/ml for SVK 14; CDDP dose 2 was 2 μg/ml for CAL 27 and 4.6 μg/ml for SVK 14; CDDP dose 3 was 3 μg/ml for CAL 27 and 6.9 μg/ml for SVK 14. For regression lines, r^2 values were comprised between 0.74 and 0.93, all P values $< 1 \times 10^{-5}$.

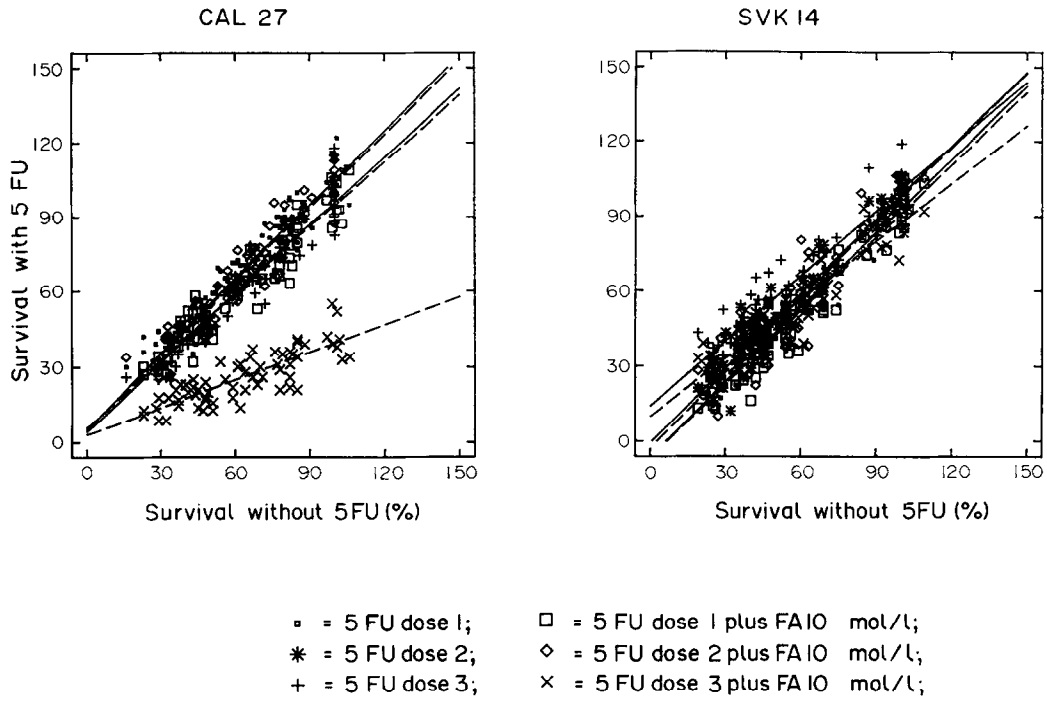

Fig. 3. Specific effect of the association 5-FU–FA within the total cytotoxicity resulting from all experimental conditions tested. 5-FU dose 1 was 2.5 ng/ml for CAL 27 and 25 ng/ml for SVK 14; 5-FU dose 2 was 5 ng/ml for CAL 27 and 50 ng/ml for SVK 14; 5-FU dose 3 was 10 ng/ml for CAL 27 and 100 ng/ml for SVK 14. The dashed lines are the regression lines for 5-FU–FA combinations and the solid lines are the regression lines for 5-FU. For regression lines, r^2 values were comprised between 0.67 and 0.95, all P values $< 1 \times 10^{-5}$ mol/l.

When considering these varying times of irradiation, the difference in the radiation effect profiles observed for CAL 27 and SVK 14 cells is not easy to elucidate. Both cells lines were sensitive to CDDP but the 5-FU–FA combination manifested a marked effect on CAL 27 cells only. This difference in chemosensitivity to 5-FU–FA association may be one explanation (Figs 2, 3). The maximal RE value for SVK 14 cells observed when irradiation is given before CDDP can be explained by postradiation enhancement. This enhancement could be explained by the inhibition by CDDP of repair of potentially lethal damage [19]. In a recent review article, Von der Maase [20] analysed the interactions of radiation and different drugs, including 5-FU and CDDP, on normal tissue and on a solid mouse mammary carcinoma *in vivo*. For normal tissue injuries, the importance of the sequence was also stressed by the author.

Keeping in mind the unavoidable more or less pronounced gap between the conclusions reached during *in vitro* studies and their clinical extrapolation, the present experimental conditions attempted to remain as close as possible to the clinical context.

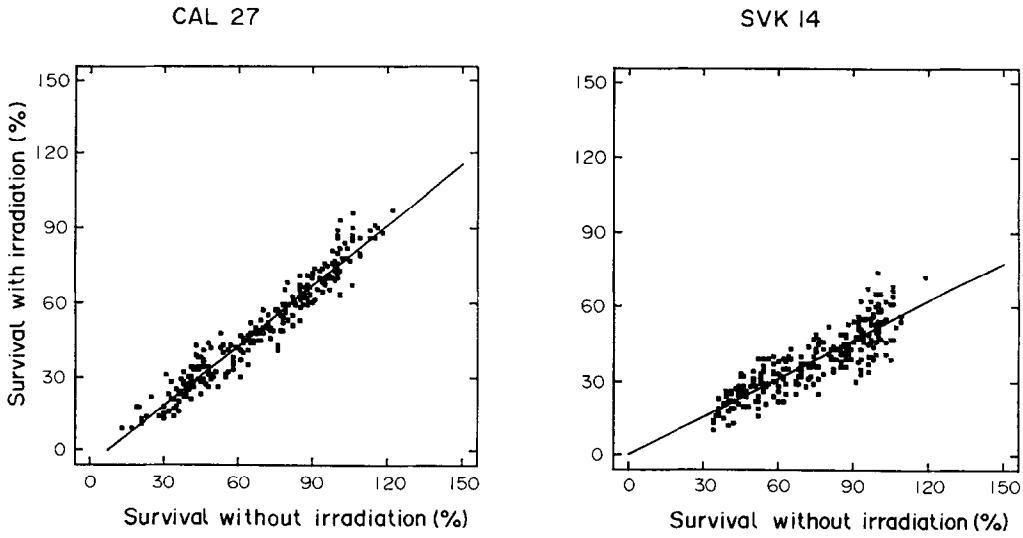

Fig. 4. Specific effect of the irradiation within the total cytotoxicity resulting from all experimental conditions tested. The radiation doses were 2 Gy for CAL 27 cells and 3.5 Gy for SVK 14 cells. For regression lines, $r^2 = 0.94$, $P < 1 \times 10^{-5}$ for CAL 27 cells and $r^2 = 0.95$, $P < 1 \times 10^{-5}$ for SVK cells.

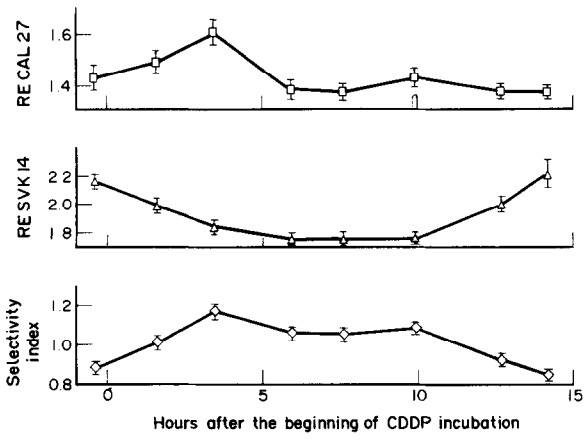

Fig. 5. Respective evolution of the radiation effect (RE) and of the selectivity index as a function of the timing for irradiation application. For definitions of RE and selectivity index, refer to the Materials and Methods section. The data represent all the 512 different experimental conditions tested; there were thus 356 RE values in total making 32 RE values per each time point tested. The vertical bars represent the standard errors. The evolution of RE values as a function of irradiation time was analysed by ANOVA: $P = 0.003$ for RE values in CAL 27 cells, $P < 0.0001$ for RE values in SVK 14 cells, $P < 0.0001$ for the selectivity index.

This was achieved by using a CDDP–5-FU sequence comparable to that used in recent protocols [21] and by exposing cells to clinically relevant drug concentrations [22, 23]. In their recent review article concerning concomitant chemoradiotherapy for patients with solid tumours, Vokes and Weichselbaum [21] emphasised the fact that cell culture experiments may provide some help in the design of clinical treatment. It is hoped the present data may fulfil this objective and open the way for future chemoradiotherapy clinical trials.

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