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In vitro combination of high dose busulfan with radiotherapy on medulloblastoma cells: additive effect without potentiation

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The aim of this in-vitro study was to evaluate the combination of busulfan with radiotherapy on TE-671 human medulloblastoma cells since unexpected clinical toxicity of busulfan was reported during the treatment of brain tumors, suggesting a possible radiopotentiation. The cytotoxicity of busulfan was determined by using colony forming assays, and doses inducing growth inhibitions of 10, 20 and 50% were selected to be tested in association: 6, 12 and 32 μmol/l for busulfan and 0.5, 1 and 3 Gy for irradiation. All possible combinations were considered within this frame and the results showed that the combination of busulfan with radiotherapy exerted an additive effect without potentiation.

Key words: Brain tumor cells, busulfan, medulloblastoma, radiotherapy.

Introduction

Busulfan is a methane-sulfonate bifunctional alkylating agent that has been in clinical use since 1959. Besides its wide clinical utility in the treatment of acute leukemia[1] and solid malignant tumors in children,[2] busulfan is expected to be a particularly valuable drug for the treatment of brain tumors because of its cytotoxic effect on cells at any stage of the cycle[3] combined with its remarkable passage in cerebrospinal fluid (CSF) with a CSF to plasma ratio of 0.95[4] when administered at high doses (16 mg/kg). It is widely used at high doses (4 mg/kg/day × 4 days) as an alternative to total body irradiation before bone marrow transplantation in children[5–7] because its late side effects are less important.

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Further to the severe cutaneous reactions recently reported,[8] that were related to radiotherapy administered soon after high dose chemotherapy including busulfan, cyclophosphamide and melphalan, questions have arisen as to potentiating effects. Since no enhanced cutaneous effect was ever observed with high dose cyclophosphamide or melphalan chemotherapy when associated with irradiation, an interaction between busulfan and radiotherapy was suggested. Cutaneous side effects were already noted with high dose busulfan[7,9] but increased toxicity due to radiotherapy has never been reported as opposed to drugs such as dactinomycin, adriamycin, bleomycin, 5-fluorouracil and hydroxyurea. This paper reports and analyses *in vitro* experiments on a human medulloblastoma cell line (TE-671) evaluating the effectiveness of the combination of busulfan with X-ray irradiation.

Materials and methods

Busulfan and radiotherapy

Busulfan, or 1,4-butanediol dimethanesulfonate (Sigma), was dissolved to a 0.1% concentration in acetone, and then kept at −20°C. Aqueous dilutions in phosphate buffered saline (PBS) were prepared from this solution.

A single dose irradiation was delivered at 250 cGy/min using a 6 MV Neptune/Therac 6 linear accelerator (CGRMeV/AECL). The culture dishes were placed on a 1 cm thick plexiglass plate in order to assure a homogeneous irradiation at the maximal dose. Dose–response curves were first drawn in order to determine the dose leading to growth inhibitions of 10, 20 and 50%. These doses were subsequently tested in association.

All possible combinations were considered and the survival rates obtained with the associations of both agents compared to their respective effects according to Steel and Peckham[10] from isobolograms: the cytotoxic effect of the combination was considered supra-additive if the surviving fractions yielded with the combined treatments were lower than the expected additivity. The term 'synergy' will be used thereinafter with this particular meaning.

Cell line and culture conditions

All culture media and additives were purchased from Seromed (Intermed, Noisy, France) except heat-inactivated fetal calf serum (FCS) that was bought from Institut J. Boy (Reims, France). Culture materials were purchased from Falcon (Grenoble, France). Stock culture of TE-671 cells was kindly provided by Dr G. Vassal (Institut Gustave Roussy, Villejuif, France) and maintained in Dulbecco MEM supplemented with 10% heat-inactivated FCS, penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere. The cultures were proved to be mycoplasma free (EORTC Clonogenic Assay Screening Study Group test procedure by Dr L. Suardet, Institut Suisse de Recherches Experimentales sur le Cancer, Lausanne, Switzerland). Exponentially growing cells were harvested by enzymatic disaggregation (trypsin/EDTA) and assayed for colony formation.

Colony forming assays

Colony forming assays were carried out by using a double layer soft agar protocol according to Hamburger and Salmon[11] with slight modifications. Briefly, 10^3 viable cells were suspended in RPMI 1640 based medium containing 0.3% agar and plated in 35 mm Petri dishes onto a basal layer made of 0.5% agar medium. Busulfan aliquots were prepared at a 10× final concentration in PBS from the 0.1% solution in acetone then diluted in the 0.3% agar complete medium. The irradiation was performed immediately after. Triplicated dishes were incubated for 14 days at 37°C to allow colony formation. Colonies with a diameter exceeding 100 μm (>50 cells) were then scored with an automatic image analysis system and compared to control untreated dishes.

Results

The radiosensitivity of TE-671 cells was determined using the multitarget model. Based on this model, the parameter D_0 determines the slope of the linear part of the curve, and indicates the dose that will reduce survival by a factor of 0.37. The extrapolation number (n_0) is derived by extrapolating the straight line portion of the curve to the survival axis. A third parameter D_q, the quasi-threshold dose, is defined as the point at which the exponential part of the curve, when extrapolated, crosses the survival level of 1.0 on the y axis. D_q is a measure of the size of the shoulder and is thought to be related to the accumulation and repair of radiation damage.

Dose–response relationships

The analysis of the dose–response curve of TE-671 cells to irradiation (Figure 1) enabled calculation of D_0 and D_q values that were found to be 1.4 and 2.5 Gy, respectively. Extrapolation number, n_0, was found to be 5.4. Doses leading to a 10, 20 and 50% growth inhibition (IC_{10}, IC_{20} and IC_{50}) were determined as 0.5, 1 and 3 Gy and selected for the following experiments. TE-671 cells were then tested for busulfan sensitivity to concentrations from 1 to 80 μmol/l (Figure 2); IC_{10}, IC_{20} and IC_{50} of busulfan were found to be 6, 12 and 32 μmol/l respectively. Acetone, which was used to dissolve busulfan, was evaluated for cytotoxicity and proved quite safe when diluted in PBS as done for the drug dilutions.

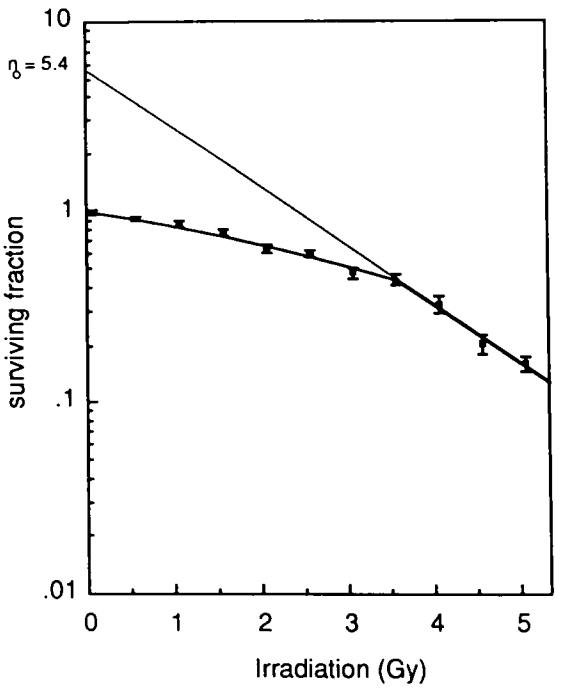

Figure 1. Dose–response of TE-671 cells to irradiation. Mean values of three triplicated experiments, bars are standard errors.

Table 1. Effect of the combinations busulfan + irradiation. The figures in parentheses show the envelop of additivity calculated from the respective survival rates obtained with each component used alone. Results are mean values of 11 triplicated experiments

Irradiation (Gy)	Busulfan (μmol/l)			
	0	6	12	32
0	1.00	0.86	0.78	0.47
0.5	0.94	0.69 (0.69/0.85)	0.59 (0.58/0.77)	0.37 (0.32/0.44)
1	0.81	0.64 (0.58/0.77)	0.54 (0.49/0.63)	0.30 (0.27/0.34)
3	0.54	0.38 (0.32/0.44)	0.28 (0.27/0.34)	0.20 (0.04/0.15)

Combination of busulfan with X-ray irradiation

All combinations between the two agents were assayed (Table 1). The expected result of each combination was calculated from isobolograms by using two modes of calculation referenced as Mode I and Mode II by Steel and Peckham[10] leading to the definition of an envelop of additivity. No synergistic (supra-additive) effect was evidenced between busulfan and radiotherapy on TE-671 cells: the results (11 triplicated experiments) only showed an additive effect since the experimental data were always within the envelop of additivity (in parentheses in Table 1).

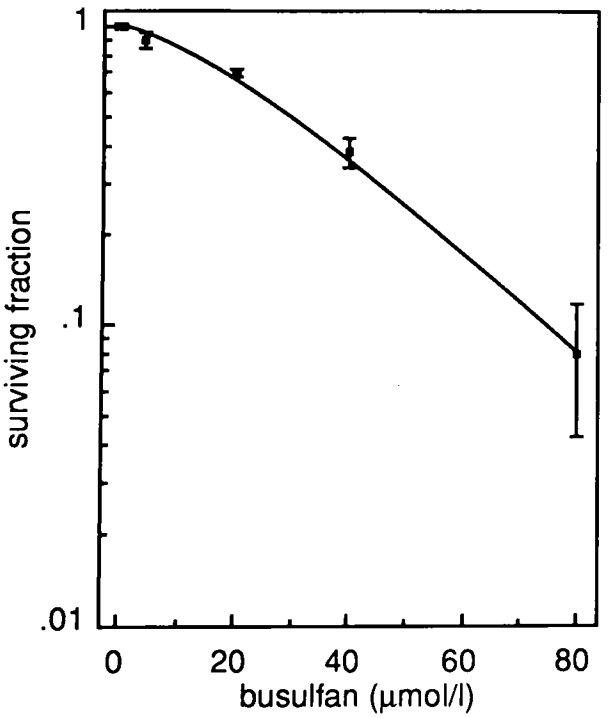

Figure 2. Dose–response of TE-671 cells to busulfan. Mean values of three triplicated experiments, bars are standard errors.

Discussion

When used at high doses as part of a marrow-ablative conditioning regimen before a bone marrow transplantation,[11] busulfan appears to be generally well tolerated though it was reported to play a part in the occurrence of convulsions.[12] The possibility of radiation potentiation that was reported by Vassal *et al.*[8] prompted us to find out if such a synergism could be observed on medulloblastoma cells in an *in vitro* study combining radiotherapy with busulfan.

The concentrations of busulfan, i.e. 4, 12 and 32 μmol/l, were selected to achieve growth inhibitions of 10, 20 and 50%. The lowest concentration (4 μmol/l) was quite pharmacologically reachable since plasma levels ranging between 1.6 and 4.4 μmol/l,[4] 1.2 and 10.4 μmol/l,[5] 1.2 and 7.2 μmol/l[6] were reported with high dose chemotherapy (1 mg/kg per os, every 6 h during 4 days) when administered before bone marrow grafting. According to the same values, the second dose (12 μmol/l) was slightly suprapharmacologic but was already reported to be reached (12.8 μmol/l) in one patient who was administered high dose busulfan at variable intervals.[6] The highest dose (32 μmol/l) was unachievable in patients undergoing high dose chemotherapy but was envisaged for experimental purposes.

These values illustrate the relative resistance of TE-671 cells to busulfan, since an effective cell-killing could not be reached by using pharmacologically relevant concentrations, and were consistent with the *in vitro* results reported by Friedman *et al.*[15] in which busulfan did not reveal any antitumor activity on mice bearing subcutaneous xenografts of TE-671 cells.

The moderate response of TE-671 cells to X-ray irradiation, with D_0, D_q, and n_0 values of 1.4, 2.5 Gy and 5.4, respectively, was consistent with the published data on the radiosensitivity of medulloblastoma cells,[14,15] reporting values of 1.3, 1.4

and 1.5, 1.6, respectively, for D_0 and n_0. The irradiation doses to be used in association with busulfan that were selected from the dose–response curve to represent IC_{10}, IC_{20} and IC_{50} values were within the range of doses currently used for *in vitro* experiments.[16] Combinations of the two cytotoxic agents were tested for additivity according to Steel and Peckham,[10] leading to the definition of an envelop of additivity which was calculated from isobolograms (iso-effects plots) because of the non-linear shape of the dose–response curves of TE-671 cells to X-ray irradiation and busulfan. When the two agents were experimentally combined, all associations produced survival rates which always laid within the envelop of additivity (Table 1), pointing out that only additive effects were yielded without any apparition of potentiation.

Conclusion

The correlation of these results with the initial clinical observation[8] indicates that the possible radiation potentiation does not seem to be attributed only to the combination of busulfan with radiotherapy. Nevertheless, further studies with other brain tumor cell lines appear to be required before any definitive conclusion can be drawn concerning the lack of synergism on brain tumors; all the more since recent molecular studies[17] tend to demonstrate that the TE-671 cell line could be referenced as a rhabdomyosarcoma cell line. The evaluation of more complex combinations including chemotherapeutic agents and radiotherapy should be put forward in order to test for ternary reactions between the various agents involved, reflecting the clinical situation of chemotherapeutic regimens.

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