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Cytotoxic and Cytogenetic Effect of Nitrogen Mustard on EUE Cells Pretreated with Sodium Warfarin

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Abstract—*A combined treatment with warfarin (W) and the alkylating agent nitrogen mustard* (HN_2) *was performed from the human heteroploid cell line (EUE). Warfarin was added to the cultures and after* 24 hr *contact the W treatment was repeated together with different* HN_2 *doses. Cytotoxicity was measured as percentage reduction in cell density and the chromosomal effects were evaluated by determining the frequency of chromosomal aberrations. As compared to EUE cultures treated only with* HN_2 *a significant increase in the toxicity values and in the amount of cytogenetic damage was found in cultures submitted to a combined treatment of* HN_2 *and W, which does not display toxicity in the experimental conditions utilized.*

INTRODUCTION

SODIUM warfarin, a well known anticoagulant agent is also used in the therapy of metastases [1, 2] since reduced blood coagulability inhibits endothelial adherence and penetration, and the fixation of tumor cells in microthrombi, which have been shown to be crucial steps in the development of metastases [3]. A series of studies has shown that adequate anticoagulation significantly reduces the incidence of metastases in experimental tumor systems and human cancer [4, 5]. With the intention of preventing metastasis formation, and of impairing tumor growth, warfarin is often associated with cytotoxic agents in cancer chemotherapy.

In this connection it appears interesting to investigate what kind of interaction, if any, occurs between warfarin and cytotoxic agents. The first compound investigated is nitrogen mustard (HN_2) chosen as representative of the alkylating agents. Both cytotoxic and cytogenetic damage to EUE cells pretreated with warfarin has been investigated and the results are reported.

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MATERIALS AND METHODS

Chromosomal preparation for evaluating cytogenetic damage

About 2.5×10^4 EUE cells/ml in 5 ml of Eagle medium with gentamycin (0.05%), supplemented with 10% calf serum, were seeded in plastic Petri dishes (on coverslips) and preincubated for 48 hr at 37°C. Five millititres of new medium containing warfarin (100 μg/ml) were then added to each monolayer and after 24 hr the cells were treated with HN_2 (see experimental design). At the end of treatment, colcemid (0.1 μg/ml) was added to each sample for 4 hr at 37°C to block the cellular mitoses in the metaphase. The chromosomes were then prepared by the method reported by De Carli and Nuzzo [6] and stained by standard methodology with Giemsa solution. A chromosomal aberration count was made on at least 100 mitoses.

Preparation of EUE cell monolayers for evaluating cytotoxic effect

One millilitre samples of cell suspension were distributed in slanted test tubes in a slow-rotating roller drum and preincubated for 48 hr at 37°C before warfarin was added. At the end of the treatment with W and HN_2

(see experimental design), each monolayer was briefly washed in Ca^{2+} and Mg^{2+} free, phosphate buffer solution (PBS), then detached by means of a trypsin solution (0.25% Difco Trypsin in PBS).

The cell suspension was counted with a Coulter counter mod. B and cytotoxic activity was calculated as described by Morasca and Leonardi [7], by measuring the inhibition of growth or destruction of the EUE cells.

The percentage of growth was calculated compared to the increase in the number of control cells. The percentage of destruction was evaluated compared to the baseline value. The experiments were repeated three times, each group consisting of five samples.

Experimental design and treatment

After 24 hr W treatment the cells were treated with W again together with HN_2 for a further 24 hr. Cells were thus exposed to W for a total of 48 hr, and to HN_2 for 24 hr. The duration of W treatment was chosen on the basis of preliminary experiments aiming at detecting the conditions giving the highest potentiating effect (unpublished data).

W was used at 100 μg/ml, i.e., the concentration which after 48 hr of contact had no inhibitory effect on cell growth. HN_2 was used at doses of 1, 5, 10 and 50 μg/ml for evaluating the cytotoxic effect and 0.005, 0.01 and 0.05 μg/ml for evaluating cytogenetic damage. The doses of HN_2 used to evaluate cytogenetic damage, were much lower than for cytotoxicity experiments since cell growth is needed to ensure cells in mitosis.

RESULTS AND DISCUSSION

The effect of HN_2 on EUE cells under W treatment was investigated by recording the number of chromosomal aberrations with and without W and HN_2 and the results are illustrated in Table 1.

Whereas W treated cells showed a frequency of abnormal mitosis comparable with the controls and HN_2 did not give rise to consistent cytogenetic damage (at least at the concentrations employed), a 4–5-fold increase in the number of mitoses with chromosomal aberrations (as compared to control and W groups) was observed when W was associated with nitrogen mustard. When HN_2 was given to pretreated cells at the dose of 0.05 μg/ml the toxicity of the treatment stopped the cells growing, and chromosomal analysis could not be made.

Table 2 shows that the cytotoxicity of HN_2 is also enhanced in the presence of W. Since the toxicity of W alone on cultured EUE cells is not significantly different from controls, doses of HN_2 in the range of 1–50 μg/ml inhibit growth or cell destruction more in W treated than in untreated cells; for instance, 1 μg/ml of HN_2 inhibits cell growth only by 50%, but when W is added, it results in cell destruction. These results, indicating that W may enhance the cyto- and geno-toxic activity of HN_2, are in good agreement with the

Table 1. Cytogenetic damage induced by different HN_2 *doses of warfarin (W) pretreated EUE cells*

Treatment (μg/ml medium)	No. of mitoses analysed	No of mitoses with aberrations*
None	100	4
W 100	100	5
(a) HN_2 0.005	100	5
(b) W 100 + HN_2 0.005	100	21
(c) HN_2 0.01	100	4
(d) W 100 + HN_2 0.01	100	23
(e) HN_2 0.05	100	14
(f) W 100 + HN_2 0.05	—	No growth

*Gaps were excluded in the count of chromosomal aberrations.
Ryan's procedure: a vs b $P<0.05$; c vs d $P<0.01$; e vs f $P<0.01$.
After 24 hr incubation with W, cells were treated with W and HN_2 together for a further 24 hr, then processed for chromosomal count as described under Methods.

Table 2. Cytotoxic activity of different HN_2 *concentrations on warfarin (W) pretreated cells*

Treatment (μg/ml medium)	Growth* (%)	Destruction† (%)
None	100	0
W 100	91	0
HN_2 1	53	0
W 100 + HN_2 1	0	12‡
HN_2 5	0	1
W 100 + HN_2 5	0	28§
HN_2 10	0	20
W 100 + HN_2 10	0	41§
HN_2 50	0	58
W 100 + HN_2 50	0	73

*Compared to control values at 48 hr ($452 \pm 9.05 \times 10^3$ cells).
†Compared to base-line values at 0 hr ($260 \pm 19.7 \times 10^3$ cells).
‡$P<0.001$; §$P<0.01$ compared to the HN_2 treated group, by analysis of variance (Duncan's test).
After 24 hr incubation with W, cells were treated with W and HN_2 together for a further 24 hr, then processed for recording growth inhibition or destruction as described under Methods.

report by Kirsch *et al.* [8] describing *in vitro* drug synergy between sodium warfarin and another antitumoral agent, 5-fluorouracil.

As to the molecular mechanism responsible for this effect, it is possible that W, which is known to bind to proteins to a high extent [9] competes with HN_2 for protein binding sites in the medium, thus increasing the amount of free compound.

It should also be considered how the reported intercalating effect of W with DNA [10, 11] is involved in this drug interaction. The fact that W, although at concentration higher than those employed in our experiments, has been described as inhibiting protein and DNA and RNA synthesis [12, 13] and as cytotoxic *per se in vitro* on cultivated human tumor cells [14] makes it tempting to speculate that the cellular and chromosomal toxicity of alkylating agents may in some way be enhanced by the presence of the compounds.

Whether other antitumoral agents behave the same way when combined with W and whether the increased activity observed *in vitro* results in a greater *in vivo* therapeutic or toxic interaction is currently under investigation.

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