The effects of epirubicin and tamoxifen on 3H- thymidine labelling index in estrogen - receptor - positive Ehrlich ascites tumor cells growing in vivo

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Abstract

The combined effect of Epirubicin (EPI) and Tamoxifen (TAM) on growth kinetics of Ehrlich Ascites Tumor cells (EAT) was investigated in vivo. In the present study, an estrogen receptor positive ER (+) hyper diploid cell lines were studied. Optimum doses of EPI and TAM (110mg and 20mg, respectively) were used. Cells were treated with these doses for 5 and 9 days. The proliferation of EAT cells growing in vivo is inhibited by EPI and TAM concentrations. The results showed that combination of EPI with TAM decreased the 3H-thymidine labeling index more effectively than did either drug alone (p<0.05). EPI+TAM treatment’s showed a maximum synergistic effect on day 5 and 9, thus inhibition of DNA synthesis was not changed by days. As a result, day factor is eliminated in combined treatment is the most important result of our study.

Key words: EAT cells, in vivo, 3H-thymidine labelling index, Epirubicin, Tamoxifen

Introduction

It is becoming increasingly likely that antineoplastic drugs may play a cytotoxic role in cell proliferation. Ehrlich ascites tumor cells (EAT), very convenient in cancer research, arised as spontaneous mammary gland carcinomas in senile female mice (Stewart et al., 1959).

Epirubicin (EPI) is the epimer of the anthracycline antibiotic doxorubicin, with inversion of the 4'
hydroxyl group on the sugar moiety, and has been used alone or in combination with other cytotoxic agents in the treatment of a variety of malignancies. EPI is a cell cycle phase non-specific anthracycline, with maximal cytotoxic effects in the S and G2 phases. Cell culture studies exhibit that EPI enters the cells rapidly, is localized in nuclei and inhibits nucleic acid synthesis and cell division (Özcan et al., 1997; Özcan Arıcan and Topçul, 2003). In the treatment of tumors, surgery, radiotherapy, chemotherapy and endocrine therapy are some times employed solely, or in a combined of two or more of them. Of the hormonal therapies available, Tamoxifen (TAM) is a semi-synthetic estrogen antagonist used in the management of pre and post menopausal breast cancer. This drug binds to intracellular estrogen receptors (ER), and prevents endogenous estrogen from binding to their own receptors. TAM is a competitive inhibitor of estradiol binding to the ER, which can cause G1 arrest in sensitive cell lines (Osborne et al., 1983). Prolonged TAM exposure blocks ER (+) cell lines in G0-G1 of the cell cycle, while it has no obvious effects on ER (-) cell lines (Levin and Chernovsky, 1989; Sipila et al., 1993). Since there have been few studies about the combined effects of EPI and TAM on cell kinetics parameters of rapidly proliferating cells. This study was conducted to understand whether the interaction of the antineoplastic drugs play role on DNA synthesis stages. We determine the in vivo cytotoxic effect of EPI and TAM and their combined effect of cytotoxicity by 'H-thymidine labelling index in EAT cells in order to increase change of these drugs in clinical applications.

Materials and Methods

Animals and EAT cells

A line of Ehrlich ascites carcinoma was supplied from the Pathology Institute of Cologne University, in Germany, and was maintained in our laboratory by weekly intraperitoneal transplantation of 6.106 cells/mouse. In the study described here, twenty male mice (BALB/c albino race) weighing approximately 20-22 g were used. The animals were kept under sterile conditions and were given food and water ad libitum. In the present study, an ER (+) hyper diploid cell lines have been studied. Test animals were divided into one control and three test groups each containing five mice.

Drug application

TAM (Tamodex, International Chemical Pharmaceutical) and EPI (Farmorubicin, Carlo Erba) were dissolved immediately before use in sterile Hank’s balanced salt solution (HBSS, Gibco Lab.) to give the required concentration. We used optimum doses of EPI and TAM (110 mg and 20 mg respectively). Cells were treated with these doses for 5 and 9 days and were exposed to EPI, TAM, EPI+TAM. At the day of 3 after inoculation, 110 mg EPI, 20 mg TAM and 110 mg EPI+20 mg TAM per 60 kg body weight were injected into animals every day during the experiment.

Estrogen receptor assay

ER levels were studied by the methods of Lippman and Huff (1976) and Raynaud et al. (1978) with minor modifications. Before the cells were injected into animals, a sample of them was separated for ER assay. The cell suspension was mixed with buffer (10mM TrisHCl, 1 mM EDTA, 12 mM thioglycerol, 3 mM sodium azide, 30% glycerol, in a 1:1 ratio) and was homogenized by a glass homogeniser for 4.5 s at 1000 rev/min. The homogenate was centrifuged at 100 000 g for 60 minutes, then 0.1 ml cytosol sample was incubated with 1 nM 3H-estradiol overnight at 40C with and without 60 nM diethylstilbestrol. After incubation, the unbound estradiol was removed by treatment with dextran-coated charcoal, and the radioactivity of the supernatant was counted in a liquid scintillation counter. Specific binding was calculated from the difference in tritium incorporated between the samples incubated with and without diethylstilbestrol. ER activity as demonstrated by the dextran-coated charcoal technique has been closely correlated with the clinical ability of TAM to inhibit tumor growth (Aydirer et al., 1997). The data were analyzed according to Scatchard graphs (Klotz, 1982). The estrogen content was found to be 12.03 fmol/mg protein. The estrogen level is characteristic of estrogen responsive tumors (McGuire et al., 1990).

'H-thymidine labelling index

On the 5th day of drug application, five mice from each group were exposed to 2µCi/g 'H-thymidine (TRA-120, Amersham, 1mCi/ml) injected peritoneal for 1 hr. After 1 hr, cell-containing ascitic fluid was
taken from animals. Drugs were continued in other experimental groups and, on the 9th day the same procedures were repeated in remaining mice.

**Autoradiography**

After labelling, cells were fixed Carnoys fixative (3: 1 ratio of ethanol: glacial acetic acid) and remaining radioactive materials were washed twice with 2% perchloric acid at 4°C for 30 mins. After preparing slides, they were coated with K.2 gel emulsion (Ilford, England) prepared with distilled water at 4°C to determine thymidine labelling index. After 3 days exposure at 4°C autoradiograms were washed with D-19 b developer (Kodak) and fixed with Fixaj B (Kodak). The slides were evaluated after being stained Giemsa for 3 mins. On each slide, with 100x12.5 magnification in 100 areas, the labelled cells were counted. The same person evaluated all the slides by counting at least 3000 cells from each animal. These data are typical results from a minimum of three independent experiments.

**Statistical analysis**

Statistical analysis of data was done using ANOVA. Dunnett’s test (between control and treatment groups) was used for multiple comparisons. For the statistical evaluation of the results, the significance was accepted at the probability level of p <0.05.

**Results**

EPI and TAM diminished the labelling index of EAT cells by increasing treatment time compared to controls. It was observed that depending on the drug treatments, inhibition of labelling index in EAT cells were increased, being of statistically significant (p<0.05) but combined treatment of EPI+TAM gave more effective results relative to the other groups (Table 1). Table reveals that treatments of EPI and TAM lower the percentage of the cells at S phase (relative to controls). When the differences of labelling index levels between different treatment groups and different times were analyzed, significant differences were found between treatments groups and between times (p<0.05). The inhibition of DNA synthesis was higher on day 9 application than those on day 5 application with drug treatments alone. Table 1 has also indicated that maximum inhibition of DNA synthesis as a function of combined treatment of two drugs.

Inhibition percentage of labelling index values, which were calculated by taking control value as 100%, was given in Figure 1. Inhibition resulted from the application of EPI alone was decreased by days, while the same action was not present in case of TAM application alone, although inhibition rate was higher. When both drugs were applied in a combination, inhibition rate was not changed by days. Thus, the fact that day parameter is eliminated in combined treatment is the most exciting result of our study.

In addition, EPI+TAM treatment’s results had a maximal synergistic effect on day 5 and 9 treatments (p<0.05). As a result, concomitant treatment combined with endocrine therapy has given improved results compared with treatment alone.

**Table 1.** 3H-thymidine labelling index values of treatment groups in EAT cells with standard deviation (±SD), statistical significance •: p<0.05.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>3H-Thymidine labelling index ±SD</th>
<th>Day 5</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.56 ± 1.09</td>
<td>13.89 ± 2.11</td>
<td></td>
</tr>
<tr>
<td>EPI</td>
<td>8.01 ± 1.23•</td>
<td>9.05 ± 3.09•</td>
<td></td>
</tr>
<tr>
<td>TAM</td>
<td>7.38 ± 1.34•</td>
<td>7.88 ± 2.23•</td>
<td></td>
</tr>
<tr>
<td>EPI+TAM</td>
<td>3.20 ± 1.97•</td>
<td>2.01 ± 1.50•</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Growth inhibition values of treatment groups calculated by taking control value as 100% in EAT cells.
Discussion

Anthracyclines including EPI, resulted in maximal cell death in the S and G2 phases of the cell cycle, but cytotoxic effects might occur in the G1 and M phases at higher drug concentrations (Plosker et al., 1993). Maximal lethal effects of EPI were demonstrated in the S and G2 phases of the cell cycle in murine and human tumor cell lines (Hill and Whelan, 1982). For many years, animal studies have been performed to study the effects of TAM on rodent mammary tumors (Gullino et al., 1975). Treatment with TAM significantly reduced the incidence of contralateral breast cancer (Cuzick and Baum, 1985). In TAM-treated patients, TAM was shown to reduce cell proliferation in human breast tumors in vivo (Clarke et al., 1993). Laboratory studies had demonstrated that chemotherapeutic cell killing was inhibited in the presence of TAM (Osborne, 1994). TAM, which can cause a G1 arrest, antagonizes the cytotoxicity of melphalan and 5-fluorouracil, but does so at dose schedules that do not affect cell proliferation (Osborne et al., 1989).

EAT cells are very convenient for the study of cell kinetic parameters. These cells actively multiply in the peritoneal cavity, so that a large number of cells in suspension are available (Stewart et al., 1959). Many studies have been performed with these cells in recent years (Mondal et al., 2002; Kennedy et al., 2002; Arimura et al., 2003; Korekane et al., 2003). In this study, a hyper diploid line was used for experiments. In the present study, adding TAM to EPI treatment had a maximal synergistic effect on day 5 and 9 (p<0.05).

3H-thymidine labelling index values obtained with the results of our previous study indicating 3H-thymidine labelling index values of 7 and 10 days are in concordance with the results of the present study (Topçul et al., 1996). Thus, the results of our study seem to be concordant with the above mentioned studies suggesting that combinations of drugs are superior to single agents. In conclusions, the data suggest that TAM treatment may modify the effect of cytotoxic combine chemotherapy in ER (+) EAT cells. The growth rate, mitotic index, continuous labelling index, cytotoxic and cell cycle effects must be examined and compared in ER-positive and negative breast cancer cell lines.

Acknowledgements

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