INTRODUCTION

1. 1. General introduction

A combination of chemotherapy and radiotherapy was applied first in the 1960s by Heidelberg, who examined the effects of 5-fluorouracil (5-FU) in combination with radiotherapy in several tumor localizations (1). There have recently been an increasing number of studies that have proved the benefit of combination therapy (2). However, the pathomechanism of such combined treatment is not completely clear.

Radiation induces many changes in tumors, and their surroundings, but just why treatment is efficient in one and inefficient in other tumor has not been explained by researchers (3).
On the other hand, the response to chemotherapy is also a complex question. It is necessary to take into account the route of administration, the drug resistance and the drug metabolism in chemotherapy. In the event of the combination of chemotherapy and radiotherapy, their effects may be additive, and interactions may also occur between.
A knowledge of the pathomechanism of such interactions is very important, since this can be utilized to maximize the tumor cell kill and to select cases where combination therapy is expected to be more efficient.

In our experimental study we set out to model the clinical situation (concomitant chemoradiotherapy) in order to demonstrate its efficiency. To potentiate the action of this treatment, we investigated tumor resistance sensitizers in combination with 5-FU chemotherapy in cell cultures. The special feature of this study is that an attempt was made to create a bridge between the clinical and the experimental scientific work, which in most cases run separately.

AIMS OF THE THESIS

Concomitant chemoradiotherapy is a new approach in cancer therapy. Randomized studies prove its superiority, but it has higher toxicity. Careful selection of the patients is essential to avoid fatal side-effect. We achieved our first experience with advanced head and neck

patients, who suffered not only the local toxicity of the radiotherapy, but also the systemic side-effects of the chemotherapy. Additionally we observed that the condition of most of these patients was too poor for them to tolerate this treatment.
Accordingly, we designed a study with a view to understanding the pathomechnism of the combined treatment in order to establish the optimum rationale schedule of chemoradiotherapy.

We planned our examinations as follows:

1. To avoid unnecessary toxicity caused by chemoradiotherapy, it is important to identify patients who have no chance of benefiting from this approach. Many genes are involved in chemotherapy and radiotherapy sensitivity. In our study, we aimed to examine Ki-67, cyclin D1, p53 and bcl-2 oncoprotein overexpression retrospectively in advanced head and neck cancer patients in order to determine relationship of its value and the tumor response to radiotherapy.

2. When cytotoxic drugs are administered with radiation therapy, it may be supposed that the two modalities interact in aa asynergistic or supra-additive way. In reality, the combined therapy can worsen the results if the chemoradiotherapy has severe additive toxicity. To analyze this interaction, we decided to model the effects of two chemotherapeutic modalities commonly used in clinical practice, i.e.5-FU, and cisplatin in combination with radiation on HEp-2 and, mouse lymphoma cell line.

3. The effectiveness of chemotherapy is limited by the emergence of multidrug tesistance. With regard to possible targets on MDR and Pgp a study was initiated to see the action of anticancer agent (5-FU and cisplatin) on MDR efflux pump.

4. There is abundant evidence that hypoxic areas within solid tumors present a barrier to effective therapy. An approach to an improvement of the effectiveness of standard therapy and circumvention of the tumor resistance brought on by hypoxia is represented by the bioreductive hypoxic-specific cytostatics. One representative such agent is tirapazamine, which has been extensively investigated in the literature and in clinical studies. We examined its antiproliferative effect in combination with 5-FU in order to compare its activity that of with vitamin C, which exhibited a chemosensitizing effect *in vitro* studies.

5. To test our theory stemming from the results of our preclinical study, we designed an investigation in advanced head and neck cancer patients, in which we focused on the locoregional control and on an assessment of acute toxicity of this combined treatment.

MATERIALS AND METHODS

3. 1. Chemicals

5-FU (Fluorouracil inj., Sigma, Ebewe, Unterach, Austria), cisplatin (Platidiam inj. La Chema, A.S., Brno, Czech Republic), vitamin C (Vitamin C inj. 10%, EGIS Pharma Rt, Budapest, Hungary), Ftorafur (Tegafur caps, Grindex, Riga, Latvia), 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MD, USA) and sodium dodecylsulfate (SDS) (Sigma Chemical Co., St. Louis, MO, USA) were used in

our experiments. Rhodamine 123 (R123) and colchicine were obtained from Sigma Chemical Co., (St. Louis, MO, USA)

3. 2. Cell cultures

The L5178Y mouse T lymphoma (parent) cell line was grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum. The HEp-2 (human epidermoid carcinoma) cell line was cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum. MRC-5 (normal lung fibroblast from human fetus) cells were cultured in RPMI 1640 medium supplemented with 10% heat-activated fetal bovine serum.

3. 3. Cell proliferation and cytotoxic assay

The antiproliferative effect and cytotoxic activity of the chemotherapeutic agents alone and in combination were tested by the MTT method. For cell proliferation assay, the drugs were diluted with culture medium in 96-well flat-bottomed culture plates in a 100 μl volume per well; 100 μl (or 50 μl) of cell suspension was added to the wells, with the exception of the medium controls. 1x104 cells /well for the mouse lymphoma (parent) cells and for the HEp-2 cells, and 1.5x104 cells /well for the MRC-5 cells were used in the cell proliferation assays. The culture plates were further incubated for 72 hours. The drug interactions were studied in combination with checkerboard methods.

In this case, the different dilutions of drug A were made in 3-12 rows in a volume of 50 μl per well and each dilution was combined with 50 μl of drug B in decreasing concentrations in the columns from A to H. The concentrations of drug A decreased from left to right and for drug B from up to down from A to H. The cell suspension was then added to the wells a volume of 50 (or100) μl containing the above-mentioned cell number. In the cell proliferation experiments, the cultures were further incubated for 48-72 hours, and at the end of the circulation period 10 μl of MTT solution (from a 5 mg/ml stock solution was added to each well (100 μl of medium). After incubation at 37 ^{o}C for 4 hours, 100 μl of SDS solution (10%) was added to each well and the plates were further incubated overnight at 37 ^{o}C.

The inhibitory effect on the cell proliferation was determined by measuring the optical density (OD). The absorbance was recorded at 540 nm; the reference wavelength was 630 nm; a Multiscan EX reader was used for evaluation. The average OD values of parallel wells of each sample and the controls were calculated. The percentage inhibitory effect or cytotoxic activity was determined according to the formula:

$$100 - \left[\frac{\text{OD sample - OD medium control}}{\text{OD cell control - OD medium control}} \right] \times 100$$

3. 4. Assay of cytotoxicity:

Wells containing $4x10^4$ mouse lymphoma cells and the monolayer cultures of adherent cell-lines (HEp-2 and MRC-5) were treated with 100 ul of medium containing the compounds at different concentrations for 24 hours. The cell viability was tested by the MTT assay and the cytotoxicity was evaluated as described above.

3. 5. Fluorescence uptake assay.

The L5178Y mouse T cell lymphoma cell line was infected with the pHa MDR1/A retrovirus. For MDR1 expression, the cells were selected by culturing the infected cells in 60 ng/ml colchicine-containing media. The L5178Y MDR cells and L5178Y parental cells were grown in McCoy's 5A medium supplemented with 10 % heat-inactivated horse serum, glutamine and antibiotics. The cells were adjusted to a density of 2 $x10^6$ /ml, resuspended in serum-free McCoy's 5A medium and then distributed as 0.5 ml aliquots in Eppendorf centrifuge tubes. The test compounds were added to the cells in quantities from 1.0 to 10 µl of the 1.0 mg/ml stock solutions and the samples were incubated for 10 minutes at room temperature. Then, 10 µl of the indicator R123 (5.2 µM final concentration) was added to the samples and the cells were incubated for a further 20 minutes at 37 ^oC. The cells were washed twice and resuspended in 0.5 ml phosphate-fuffered saline for analysis. The fluorenscence of the cell population was measured by flow cytometry with the Beckton Dickinson FACScan instrument.

3. 6. Histological analysis

The tumor samples were fixed in neutral 10% formalin, dehydrated and embedded in paraffin. 4 μ tissue sections stained with hematoxylin-eosin were prepared for routine histological examination.

Automated immunohistochemistry (Dako Techmate ^{TM} 500 Plus, Dako, Glostrup, Denmark) was carried out according to the streptavidin-biotin-peroxidase technique. The sections were incubated with commercial primary monoclonal antibody against p53 oncoprotein (Clone DO7, 1:100), bcl-2 oncoprotein (Clone 127, 1:200), cyclin D1 (Clone DCS 6,1:80), Ki-67 (1:1000) from Dako (Glostrup, Denmark) and MDR (Clone JSB1, 1:50) from Biogenex. Appropriate positive and negative controls were included in each run.

3. 7. Irradiation

Experimental study

We irradiated the cell cultures in microplates by means cobalt 1.25 MV machine.

Clinical study

Study I (Radiotherapy alone)

We administered a standard dose of 70 Gy of conventional fractionated radiotherapy. Radiotherapy was delivered with a cobalt 1.25 MV machine, using a three-field technique with two lateral coaxial fields, including the primary and upper neck nodes and an anterior lower neck and supraclavicular field . All patients received 50 Gy to this lower volume following a 2 Gy daily dose schedule. The spinal cord was protected after 40 Gy. The total dose to the primary site and metastatic lymph nodes was 66-70 Gy.

Study II (Chemoradiotherapy)

Chemotherapy (Ftorafur) was administered every day throughout the radiotherapy in an oral dose of 30 mg/m^2 2 times per day.

3. 8. Patient selection

To be considered eligible for this study, patients had to meet the following criteria: stage III or IV (International Union Against Cancer [UICC] criteria) squamous cell unresectable

carcinoma of the oral cavity, oropharnx, hypopharynx or larynx or carcinoma of unknown origin with cervical metastatic nodes.

Patient group I: Between 1998 and 2001, 33 patients (2 females and 31 males) were observed. The primary aim of the study was to evaluate the radiosensitivity of the tumors in accordance with the genetic alterations.

Patient group II: Between November 2000 and January 2003, 50 patients (13 females and 37 males) were enrolled into the study. The main goal of the study was to evaluate the remission rate and toxicity of chemoradiotherapy. The patients were stratified according their performance state (Eastern Cooperative Oncology Group [ECOG]): groups 0-2, and 3-4.

3. 9. Statistical analysis

For the comparision of mean values, the *t*-test (31, 32, 33) and one-way analysis of variance were used, together with the Mann-Whitney and Kruskal-Wallis tests (31, 32, 33) in cases of non-normality. The normal distribution of the samples was tested by using Kolmogorov-Smirnov test (31). The Spearman correlation coefficient (31, 32, 33) was applied to assess correlations between continuous variables. Survival curves were constructed by the Kaplan-Meier method (32, 33) and compared by using the long-rank test. The dependence of the survival curves on the predicting factors was analyzed by means of Cox regression (proportional hazard model) (32, 33). The cut-off values for the categorization of the predicting factors were chosen so as to ensure the appropriate case numbers for the statistical procedures. To evaluate the possible correlation between the condition of the patients and the tumor response, we calculated Tschuprow coefficient (34).

RESULTS

4. 1. Evaluation of predictive factors

33 patients with advanced head and neck cancer were analyzed as concerns the remission rate and survival. Analyzing the effects of all four predicting factors, only the bcl-2 levels resulted in a significant difference (Table II).The estimated survival curves for the bcl-2-positive and negative groups clearly indicates a better survival for the bcl-2-positive group (Fig. 1), but the

survival curve for this group is less reliable because of the small number of bcl-2-positive cases. Although the correlation is significant, more data acquisition is required for a more precise comparison.

4. 2. Preclinical results with a combination of irradiation and chemotherapy

We found that in the case of HEp-2 cells even extremely high doses of irradiation hardly affected the growth rate of the HEp2 cells.However in the case of mouse lymphoma cells relatively low dose of radiation achieved high tumor growth inhibition(Fig. 2.3). Based our results we considered the mouse lymphoma cells as radiosensitive, while HEp-2 cells as radioresistant cell line.

Combined effect of chemotherapy (Cisplatin and 5-FU) and radiation was examined on both cell lines (mouse lympoma PAR and HEp-2 cells).

Cisplatin alone exerted a dose-dependent inhibitory effect on the cells. When the optimum dose of irradiation was combined with various concentrations of cisplatin, a noteworthy, synergistic increase in growth inhibition was found in the case of the drug-sensitive PAR cells at a low level of cisplatin (fig.4.)

We also examined the combined effect of 5-FU and irradiation.

The effect of the combination of 5-FU and irradiation on the mouse lymphoma PAR cells was dose-dependent, and the additive effect of radiation and 5-FU was similarly more relevant in the lower 5-FU concentration range than at higher concentrations (Fig.5).

As regards the HEp-2 cells, only high doses of chemotherapy influenced the inhibition of tumor cell growth. In addition, increasing doses of chemotherapy (cisplatin and 5-FU) in combination with irradiation did not change the effect of irradiation (Fig.6.7)

4. 3. MDR reversal effect of Cisplatin and 5-FU

In a control experiment we found that the MDR reversal effect of the two tested drug did not change the accumulation of the human MDR1 infected mouse lymphoma cells (Table III).

4. 4. Preclinical results with tumor response modifier drugs in combination with 5-FU

A synergistic interaction of tirapazamine in combination with 5-FU was observed for the antiproliferative effect both in the mouse lymphoma cells and in the HEp-2 cell line (Fig.8,9). However, the antiproliferative effect of 5-FU potentiated by tirapazamine was higher in the case of the mouse lymphoma cell line, than in the case of the radioresistant HEp-2 cells.

In a search for a clinically available agent that can be administered orally we found that vitamin C may act as a chemomodulator, potentiating the cytotoxic activities of various chemotherapeutic agents.

We found that different doses of vitamin C in combination with 5-FU chemotherapy had no effect on the normal fibroblast (MRC-5)cells (Fig.10.).

On the other hand we found, that vitamin C increased the anticancer effect of 5-FU in a dose-dependent manner. In the case of mouse lymphoma cell line 5-FU in combination with vitamin C proved to have an increased antiproliferative effect relative to that of 5-FU alone only when the vitamin C concentration was above 5 ug/ml. A lower dose did not modify the antiproliferative effect of 5-FU (Fig.11.).

For the radioresistant cell line HEp-2, low doses were ineffective, but high doses (higher than 50 μg/ml) of vitamin C markedly enhanced the antiproliferative effect of 5-FU (Fig. 12.)

4. 5. Clinical results in patients treated with chemoradiotherapy

we designed a study involving an orally administered well-tolerable drug (Ftorafur) in order to establish the treatment outcome in two patient groups: with good (ECOG 0-2) or poor (ECOG 3-4) performance states. Since the survival in these patient groups depended on many other factors, such as the nutrition, the patient's life style, the alcohol- drinking habits, the immune status etc., we focused our investigation only on the local control of the treatment which correlated best with symptom relief.

Response

The overall response ű rate for the 50 patients was 94% (95% confidence interval [CI] 88 to 99). A complete response was achieved in 60% (95%, [CI] 48 to 72) (Table IV).

Acute toxicity

The 50 patients were evaluated for acute toxicity in 2 groups (good and poor performance status) (Table VI). The hematologic toxicity was grade 3 in only 1 case in the good performance status group, and in 3 cases in the poor performance status group. The same patients exhibited gastrointestinal toxicity of grade 2-3. All the symptoms could be quickly

eliminated by hydration and careful supportive care. Febrile neutropenia and sepsis were not experienced, but 1 patient died soon after the completion of the therapy, from tumor bleeding.

Mucositis was the dominant toxicity; it occurred in grade 2 in 44% of the patients, and in grade 3 in 12%. Skin toxicity was moderate: only 9 (18%) of the patients developed a moist desquamation. Loss of >10% of the initial body weight occurred in 6 patients (12%).

DISCUSSION

5. 1. Importance of predictive factors

The only examined factor which displayed significant corrrelation with the survival in our study, was bcl-2 in our study. It has recently been reported that bcl-2 appears to be a new class of prognostic indicator which, although a marker of advanced disease, defines a relatively small population (12.8 %) of such head and neck cases that have a favorable outcome independently of the treatment (38).

Our results demonstrated that the examined molecular biological markers did not allow the selection of patients who would respond worse or better to radiotherapy. We consider that tumor progression is a complex event with many forms of mutations, which cannot be characterized by one or several genetic alterations. In conclusion, we believe that the bcl-2 protein is a fascinating molecule which may play a role in the therapeutic strategy for cancer in the near future.

2. Preclinical study with combined chemoradiotherapy

We assume that concomitant chemoradiotherapy can be more effective than either single-treatment modality in the case of radiosensitive tumors, if continuous low-dose chemotherapy (cisplatin and 5-FU) and radiation are applied simultaneously. The continuous use of chemotherapeutic agents might serve to rescue the optimum cell cycle synchronization and consequently cell populations more sensitive to radiation. In the case of chemoradioresistant tumors, only very high doses are effective where the induced damage is nonrepairable. The effectiveness of high doses of radiation and high doses of chemotherapy in the event of therapy resistance can be explained by the higher rate of lethal cell damage, which is unrepairable (33). In this case the benefit of combined therapy is less, since fewer repairable lesions and fewer surviving clones exist.

5. 3. Effect of Cisplatin and 5-FU on the MDR efflux pump

The majority of anticancer drug (like Cisplatin and 5-FU) and intervention (like ionizing radiation) do not inhibit MDR but may induce the expression of the MDR1 gene ®. Taking into account these findings combination of chemotherapy and radiotherapy could not overcome MDR resistance, but may induce further expression of MDR1 gene. Therefore new targets and treatment strategies (combination anticancer treatments with P-glycoprotein decreasing agents) should be find to overcome tumorresistance.

5. 4. Chemosensitizing effect of tirapazamine and vitamin C

Nutritionally deprived hypoxic tumor cells are resistant to conventional chemotherapy or radiotherapy in consequence of their non-cycling status (G0 phase), limited drug diffusion, and limited oxygen perfusion (39, 40, 41). Treatment with these agents alone leaves the hypoxic regions of tumors unharmed. Viable hypoxic cells that survive cytotoxic therapy may result in cell proliferation and tumor regrowth, and also in mutation and the development of resistance.Agents which selectively target hypoxic cells may enhance the antitumor efficacy of chemotherapy and radiotherapy.

The antitumor activity of tirapazamine has been attributed to its ability to damage DNA. Specifically, the damage is attributed to a free radical which is formed enzymatically via a one-electron reduction of tirapazamine. In the presence of oxygen, the free radical is spontaneously oxidized back to the parent drug. However, in poorly oxygenated (hypoxic) cells, back-oxidation is limited, allowing the free radical to damage cellular components, including DNA.

Similarly to the result of the experiments with tirapazamine, we found significant enhancements of the antiproliferative effect of vitamin C in combination with 5-FU chemotherapy in mouse lymphoma and HEp-2 cell cultures. As in the case of tirapazamine, lower doses of vitamin C could achieve the same antitumor effect in mouse lymphoma cells as high doses of vitamin C in combination with 5-FU chemotherapy in the chemo- and radioresistant HEp-2 cell line. Assuming that vitamin C can operate via electron transfer, we explain the results in terms of the oxidizing effect of vitamin C.

5. 5. Results on patients

We assume that our good results could be explained in a part by the permanent interaction between the radiation and chemotherapy in consequence of the daily oral chemotherapy schedule, and the individually determined tolerable chemotherapy dose. Since the therapy

does not involve extra toxicity, the full-dose radiation schedule could be administered without long breaks to almost every patient.

We succeeded in demonstrating the possibility of achieving high antitumor activity with orally administered continuous low-dose chemotherapy in combination with concomitant radiotherapy. The major advantages of this approach are the easy administration, and the tolerability for all patients. Future studies should evaluate the effects of supplementary and maintenance therapy on the survival.

CONCLUSIONS

In order to select patients with radiosensitive tumors, we searched for molecular markers with which to predict the treatment outcome in patients with advanced squamous cell carcinoma of the head and neck. We investigated the effects of chemotherapy (5-fluorouracil and cisplatin) in combination with irradiation in cell cultures (mouse lymphoma and HEp-2 cell line). To improve the effect of chemoradiotherapy, we investigated the use of drugs (tirapazamine and vitamin C) to overcome tumor resistance. These were tested in patients with advanced head and neck cancer.

To summarize our results:

1. The examined predictive factors (Ki-67, cyclin D1, p53 and bcl-2 overexpression) in advanced head and neck cancer point to the high proliferating activity of these tumors, but the heterogeneity of our results do not allow us to conclude that these parameters are of prognostic value. However the high proliferating activity of these tumor should take into account in the treatment strategy (e.g.:starting anticancer treatment as soon as possible, applying hyperfractionated chemoradiotherapy, without long breaks may improve treatment outcome).

2. Our experimental study releated that 5-fluorouracil(5-FU) and cisplatin chemotherapy in combination with radiotherapy show synergistic effect in in case of (mouse lymphoma cell) line, but had no additive effect on HEp-2 (radioresistant) cells.In sensitive cases low dose of chemotherapy was sufficient for sensitisation the radiation effect.

3. Anticancer treatments (chemotherapy and radiation) do not inhibit MDR, but further increase the expression of MDR1 gene. Therefore combination these treatments with P-glycoprotein decreasing agents would be mandatory.

4. Searching for agents to overcome tumor resistance by other pathomechanism than MDR we found that vitamin C enhanced the antiproliferative effect of 5-FU chemotherapy- in vitro in chemomsensitive mouse lymphoma and resistant HEp-2 cell line as well, although only high doses of vitamin C can sensitize the effect of 5-FU chemotherapy in case of hypoxic, chemoresistant tumor cells (HEp-2). The results were comparable with the antiproliferative effect of the hypoxia-selective agent tirapazamine, suggesting that vitamin C may influence the tumor oxidative status.

5. In our clinical study we found that continuous administration of the oral 5-FU derivate (tegafur) in combination with radiotherapy is efficiant treatment schedule in respect of locoregional control, and has no additive general toxicity.

ACKNOWLEDGMENT

I am very grateful to **Prof. József Molnár** for offering me the possibility to work in his laboratory. He aroused my interest in the experimental work, and gave me the pleasure of success. He understood my clinical problems and helped me to put these into a laboratory setting, and to find solutions. Through his openmindness, provided me with excellent support.

I express my ever-lasting gratitude to **Prof. Ilona Mucsi** for her continuous support of my laboratory work, for her and constructive guidance.

I would like to express my grateful thanks to **Prof. László Thurzó,** who ensured the conditions for my research work beside my clinical work.

Last but not least, all my co-workers, colleagues and staff members at the Departments of Microbiology and Oncotherapy, Faculty of Medicine, University of Szeged are gratefully thanked for creating a supportive and pleasant working environment.

This thesis was financially supported by the Foundation for Cancer Research of Szeged (Szegedi Rákkutatásért Alapítvány).

I dedicate my thesis to my patients.

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