Introduction

Head and neck squamous cell carcinomas (HN-SCC) are the most common types of tumours appearing in the upper aerodigestive tract. They may occur in a variety of tissue types and within a wide malignancy range. Head and neck carcinomas account for about 4% of all new cancer diagnoses and about 2% cancer deaths. According to the American Cancer Society, in 2008 it was expected that there will be 47,560 diagnosed cases of oral cavity, pharyngeal and laryngeal neoplasm (Jemal et al., 2008). The etiology of head and neck carcinomas is associated with many agents. Tobacco smoking is the most common risk factor associated with HNSCC (Freedman et al., 2007). Moreover, alcohol consumption increases its carcinogenic effect (Maier et al., 1992). Other factors implicated in the carcinogenesis of upper aerodigestive tract tumours include oncogenic viruses (D’Souza et al., 2007; Sisk et al., 2003) and genetic instabilities (Gleich and Salamone, 2002; Zhou et al., 2007).

The aim of our research was to estimate the sensitivity of head and neck cancer cells to genotoxic treatment with $\gamma$-radiation and $\gamma$-radiation used in anticancer therapy. Hydrogen peroxide was used as an internal control of DNA damages after genotoxic treatment of cells from HNSCC patients.

DNA repair is critical for successful chemo- and radiotherapy of human tumours, because their genotoxic sensitivity may vary in different types of cancer cells. In this study we have compared DNA damage and the efficiency of its repair after genotoxic treatment with hydrogen peroxide, cisplatin and $\gamma$-radiation of head and neck squamous cell carcinoma (HNSCC). Lymphocytes and tissue cells from biopsies of 37 cancer patients and 35 healthy donors as well as the HTB-43 larynx cancer cell line were employed. The cell sensitivity to genotoxic treatment was estimated by the MTT survival assay. The extent of DNA damage and efficiency of its repair was examined by the alkaline comet assay. Among the examined treatments, we found that HNSCC cells were the most sensitive to $\gamma$-radiation and displayed impaired DNA repair. In particular, DNA damage was repaired less effectively in cells from HNSCC metastasis than healthy controls. In conclusion, our results suggest that the different genotoxic sensitivity of HNSCC cells may depend on their DNA repair capacity what in turn may be connected with the effectiveness of head and neck cancer therapy.

Key words: Head and Neck Cancer, DNA Damage, Comet Assay
Interestingly, recent data evidenced that cancer cells are more susceptible to \( \text{H}_2\text{O}_2 \)-induced death than normal cells (Caldecott, 2001). Cisplatin is one of the most common drugs used in anticancer therapy for over 30 years. It is a kind of cytostatic alkylating-like compound which causes DNA inter- and intrastrand cross-links, mainly with purine bases (1,2-GpG, 1,2-ApG or 1,3-GpNpG), and it may also induce DNA-protein cross-links (Chválová et al., 2007; Weijl et al., 1998). DNA damages caused by CDDP are mainly repaired by nucleotide excision repair (NER) which contributes to the lower sensitivity of cancer cells to this drug (Kartalou and Essigmann, 2001). Thus, cisplatin is often combined with radiotherapy, which increases the treatment efficiency. Radiation alone is usually offered to patients at an early stage and for locoregionally advanced (stage III or IV) head and neck cancer. The conventional dose used in radiation therapy is approx. 2 Gy daily. It has to be noticed that \( \gamma \)-radiation is the most potential DNA-damaging agent which causes SSBs and DSBs, base and sugar modifications, AP sites, cross-links and others. DNA damages after \( \gamma \)-radiation treatment can be repaired by NHEJ, HRR or BER pathways (Gulston et al., 2004).

DNA lesions have to be repaired efficiently to maintain genome integrity, what is essential for proper cell function. Defects in the DNA repair pathway may affect the cell sensitivity to DNA damage resulting in cell death or future cancer development. The knowledge about the range of lesions caused by DNA-reactive drugs and tumour response may be useful to estimate the effectiveness of anticancer treatment. It may indicate which of the factors used in trials has the strongest effect on cancer cells and which side effect on healthy cells is presented. In order to estimate the DNA damage and repair efficiency after treatment of cells with hydrogen peroxide, cisplatin and \( \gamma \)-irradiation we used the comet assay analysis. We examined peripheral blood lymphocytes, cells isolated from primary tissue biopsies and metastasis of HNSCC patients. Lymphocytes and tissue cells from healthy donors were used as controls. HTB-43 larynx cancer cells were employed as controls for squamous carcinoma.

**Material and Methods**

*Study subjects*

Thirty seven patient subjects [19 men and 18 women, mean age (56 ± 7)] with HNSCC were enrolled in the study. Most of the patients have been diagnosed with larynx cancer, 16 among the patients had metastases of HNSCC (neck, tongue, salivary glands, and tonsils). The cells from 35 subjects without cancer [18 men and 17 women, mean age (52 ± 11)] were used as controls. The tissue samples and lymphocytes were collected from the patients before they had received any chemotherapy or radiation therapy for their primary disease. The diagnosis of healthy and tumour tissue was applied after histopathological examination of patient biopsies. Normal tissues obtained from patient biopsies during standard medical examination, in order to exclude cancer possibilities, were used as controls. These subjects without cancer were also used as blood donors. Moreover, controls were selected based on family history, in order to exclude familiar predisposition to cancer development. Prior to examination, the patients and control subjects did not receive other medicaments such as antibiotics or steroids. Despite a 4 years younger control, there were no statistical differences in the age of analyzed patient subjects and the control group (\( P = 0.068 \)). Patient and control subjects enrolled in the examination were non-smokers. Another exclusion criterion for the patient and control subjects enrolled in the examination was alcohol consumption. All patient and control subjects were recruited from two medical units: Department of the Head and Neck Neoplasm Surgery and Department of Otolaryngology and Oncology, both of the Medical University of Lodz, Lodz, Poland. All subjects included in the study were unrelated Caucasians and lived in the Lodz district, Poland. The study was approved by the Local Ethic Committee and a written consent was obtained from each patient or healthy blood donor before enrolling into the study. The HTB-43 larynx cancer cell lines commercially available (ATCC, USA) were used as controls for squamous cancer cells isolated form tissue biopsies of patients with head and neck cancer. HTB-43 is a well characterized cell line of squamous cancer; therefore it is an appropriate control for tissue biopsy analysis.
Cell treatment

The research material consisted of several types of cells such as: HNSCC cells taken from primary tumours, cells from metastasis biopsies, and cells taken from non-oncogenic tissue, HTB-43 cell line, blood lymphocytes isolated from HNSCC patients and from healthy donors. Fragments of isolated tissues were treated by solution containing proteinase K, collagenase and HBSS (Hank’s buffered salt solution), then incubated to obtain a cell suspension. The HTB-43 larynx cancer cell line was cultured in EMEM (contained also 10% fetal bovine serum and antibiotics: penicillin, streptomycin). It was kept at 37 °C in an atmosphere containing 5% CO₂. Lymphocytes were isolated from peripheral blood of HNSCC patients and healthy donors by centrifugation in a Histopaque-1077 (Sigma) instrument.

MTT assay

Cell suspensions (1.5 · 10⁶/ml of growing medium) after hydrogen peroxide, cisplatin or γ-radiation treatment were incubated for 3 d. Then the cell viability was evaluated by the MTT assay. Cells were plated onto 96-well plates in 150 µl of growth medium, and 20 µl of 10 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent were added to each well. After incubation at 37 °C for 4 h, the supernatant was removed and 200 µl of a solution containing 10% SDS and 0.04 M HCl were added to dissolve the formazan salt. 1 h later the difference of absorption intensity, (OD₆₅₀ nm − OD₅₇₀ nm), was measured by an ELISA microplate reader (Bio-Rad, Herkules, CA, USA). Results are presented as percentage of viable cells.

Comet assay

In order to detect the DNA damage level and repair efficiency the single cell gel electrophoresis (SCGE) assay was performed. The assay was done under alkaline conditions according to the procedure of Singh et al. (1988) with modifications by Klaude et al. (1996). Cells were suspended in 0.75% low-melting agarose dissolved in PBS and layered onto microscope slides (Superior, Germany) previously covered with 0.5% normal-melting agarose. Lysis of the cells was undergone at 4 °C for 1 h in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM tris(hydroxymethyl)aminomethane, pH 10. Next, the DNA was unwound and uncoiled under alkaline conditions (pH > 13) for 40 min in the electrophoretic solution consisting of 300 mM NaOH and 1 mM EDTA. Electrophoresis was carried out at 4 °C (the temperature of the running buffer did not exceed 12 °C) for 30 min at an electric field strength equal to 0.73 V/cm (30 mA). Preparations were then done for the neutralization with 0.4 M tris(hydroxymethyl)aminomethane, pH 7.5. Next, cells were stained with DAPI solution (2 µg/ml), covered with a cover glass and incubated at 4 °C for 45 min. All of the steps described above were performed under dimmed light or in the dark in order to prevent additional DNA damage.

DNA cross-links assay

The analysis of repair of cross-links generated by cisplatin was performed as described previously for the comet assay procedure with modification made by McKenna et al. (2003). After cisplatin post-treatment repair incubation, the cells were irradiated to induce strand breaks with 15 Gy radiation at the dose rate 0.017 Gy/s using a 60Co source. In each experiment, controls were prepared with cells, which were not treated with cisplatin and subsequently received either no radiation (negative control) or 15 Gy radiation only (positive control). After irradiation, the cells underwent the standard procedure described above. In this modified procedure the comet tails increased with post-repaired treatment incubation time revealed repair response to cross-linking agents. This modified procedure showed the efficiency of cross-links repair after irradiation by the percentage of DNA in the comet tail.

Comet analysis

The objects were observed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, San Diego, CA, USA) equipped with a UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to the personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Prague, Czech Republic). Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells, and the mean percentage of DNA in the comet tail (tail DNA %) was calculated. This quantity is positive-
ly correlated with the level of DNA breakage in a cell and was taken as an index of DNA damage in each sample.

Statistical analysis

The data of the MTT test were expressed as mean ± SD and the values of the comet assay were expressed as mean ± SEM. Three separate experiments for each patient with control were performed and statistically analyzed. If no significant difference between variations was found, as assessed by the Snedecor-Fisher test, the difference between means was evaluated by applying Student’s t-test. Otherwise, the Cochran-Cox test was used. The data was analyzed using Statistica (StatSoft, Tulsa, OK, USA) statistical package.

Results

Cell viability

Table I shows the mean percentage of the viability of lymphocytes from healthy donors used as a control, lymphocytes from HNSCC patients and HTB-43 cells measured by the MTT test after 3 days of incubation with an increasing concentration of hydrogen peroxide from 2.5 to 50 μM or cisplatin from 0.05 to 10 μM, or irradiated with an increasing dose of γ-radiation from 5 to 50 Gy. It was found that HNSCC cells were more sensitive to genotoxic treatment than control cells. With the highest dose of hydrogen peroxide, 50 μM, the viability of the control cells was 22% and for lymphocytes from HNSCC patients the average was 6%. The viability of the lymphocytes from HNSCC patients for the cisplatin concentration of 0.5 μM decreased to 86% and to 28% for the concentration of 10 μM as compared to the control (95% and 52%). The viability of the control cells for 25 Gy was 90% and for 50 Gy it was 79%, while for lymphocytes from HNSCC patients the average viability was 84% and 55%. The average viability of the HTB-43 cells after genotoxic treatment was also lower than that of control cells from healthy donors.

DNA damage

Fig. 1 shows DNA damage measured as mean percentage of DNA in the tail comet (Tail DNA %) of lymphocytes form healthy donors, lymphocytes from HNSCC patients and HTB-43 cells (left panels), and healthy tissue cells, HNSCC tumour cells and cells from metastasis (right panels), after genotoxic treatment with increasing concentrations, from 0 to 25 μM, of hydrogen peroxide or 0 to 10 μM cisplatin, or irradiated with increasing doses from 0 to 50 Gy. All types of analyzed cells were sensitive to genotoxic treatment. There were statistically significant differences in the genotoxic susceptibility of patient and control lymphocytes to hydrogen peroxide at 2.5 μM (P<0.05), 5 μM (P<0.001) and 15–25 μM (P<0.001) and to cisplatin at all concentrations (P<0.001), except for the lowest cisplatin concentration (P>0.05), as well as to γ-radiation at any used dose from 5 to 50 Gy (P<0.001). Hydrogen peroxide treatment induced statistically significant differences in DNA damages of HTB-43 cells, patient cells from primary tumours and healthy controls at concentrations from 10 μM to 15 μM (P<0.05) and at 25 μM (P<0.001), as well as patients cells from HNSCC

Table I. The viability of peripheral blood lymphocytes from HNSCC patients, HTB-43 cells, and healthy donors measured by the MTT test after 3 days of incubation with an increasing concentration of hydrogen peroxide from 2.5 to 50 μM or cisplatin from 0.05 to 10 μM, or irradiation with increasing doses of γ-radiation from 5 to 50 Gy.

<table>
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<tr>
<th>H2O2</th>
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<td>Concentration [μM]</td>
<td>control</td>
<td>HNSCC</td>
<td>HTB-43</td>
<td>Concentration [μM]</td>
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<td>6***</td>
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Error bars denote SD ( * P<0.05; ** P<0.01; *** P<0.001 according to control).
metastasis ($P<0.001$) of all concentrations from 5 to 25 $\mu$M ($P<0.001$). The highest percentage of tail DNA was observed in tissue cells from patients with metastasis of HNSCC. For cisplatin treatment the highest percentage of tail DNA was observed in HTB-43 cells, however, there were no statistically significant differences in the genotoxic susceptibility to cisplatin between cells from tissue biopsies of healthy subjects, HNSCC patients and HNSCC metastasis ($P>0.05$). For $\gamma$-radiation treatment the DNA damage level was higher in patient cancer cells than in healthy controls. There were statistical differences in the genotoxic susceptibility of patient and control lymphocytes to $\gamma$-radiation from 15 to 35 Gy ($P<0.001$) and patient cells from primary tumours and healthy controls to $\gamma$-radiation at 25 and 35 Gy ($P<0.01$), as well as patient cells from metastasis ($P<0.01$) and HTB-43 cells at 15 Gy and 35 Gy ($P<0.001$) in comparison to healthy controls.

**DNA repair**

Figs. 2, 3 and 4 show the time course of DNA repair measured as a mean percentage of DNA in the comet tail (Tail DNA %) lymphocytes from healthy donors (A), lymphocytes from HNSCC patients (B), HTB-43 cells (C), healthy tissue cells (D), HNSCC tumour cells (E), and cells from metastasis (F), after genotoxic treatment. Our experiments were based on using two different concentrations, 5 and 10 $\mu$M, of hydrogen peroxide and two concentrations, 1 and 5 $\mu$M, of cisplatin with post-treatment of 15 Gy irradiation, or irradiation with two doses 15 and 25 Gy. Cells were taken for analysis immediately after exposure and after 30, 60, 120 and 240 min of repair. In all cases, DNA repair...
damage in the control cells was constant, which indicates that the preparation procedure did not induce significant DNA damage. Fig. 2 shows that lymphocytes from healthy donors (A) and lymphocytes from HNSCC patients (B) treated with 5 and 10 μM of hydrogen peroxide were able to repair DNA damage completely \((P > 0.001)\) during 120 and 240 min post-treatment incubation repair. HTB-43 cells (C) treated with 5 or 10 μM of hydrogen peroxide were not able to complete repair even after 60, 120 or 240 min post-treatment incubation repair \((P < 0.001)\). At 5 or 10 μM concentration and after 60, 120, 240 min of incubation repair healthy tissue cells (D) and HNSCC tumour tissue cells (E) were able to repair DNA damage completely \((P > 0.001)\). At 5 or 10 μM of hydrogen peroxide treatment and 120, 240 min \((P > 0.001)\), 60 min \((P > 0.01)\), 30 min \((P > 0.05)\) post-treatment incubation repair DNA damage repair was displayed in cells from metastasis (F). There was no statistically significant difference \((P > 0.001)\) in peripheral blood lymphocytes from healthy donors (A), lymphocytes from HNSCC patients (B), HTB-43 cells (C), healthy tissue cells (D), HNSCC tumour cells (E), and cells from metastasis (F) treated with 1 or 5 μM of cisplatin compared to the DNA damage level in control cells after 240 min of post-treatment incubation repair (Fig. 3). Lymphocytes from healthy donors (A) exposed to 15 and 25 Gy were able to repair DNA damage completely \((P > 0.05)\) during 240 min of post-treatment incubation repair. Doses of 15 and 25 Gy caused DNA damage that was not repaired by lymphocytes from HNSCC patients (B) and
HTB-43 cells (C) after 240 min post-treatment incubation repair ($P<0.001$) (Fig. 4). We also found that cells from healthy tissue biopsies (D) were able to repair DNA damage within 240 min after $\gamma$-radiation treatment at doses of 15 and 25 Gy ($P>0.05$). In contrast, HNSCC tissue cells (E) and HNSCC tissue biopsy cells from metastasis (F) did not repair damage within 240 min of post-treatment incubation repair at doses of 25 Gy ($P<0.05$) and 15 Gy ($P<0.01$), 25 Gy ($P<0.001$), respectively. In all cases, DNA damage in the control cells was constant, indicating that preparation and subsequent processing of the cells did not implicate significant damage to their DNA.

**Discussion**

The tumour response to chemo- and radiotherapy may on depend on the cancer cells’ ability to repair DNA damage. Genotoxic sensitivity to DNA-reactive drugs and radiation may vary among different types of cancer cells. One of the most important tasks of molecular oncology is to identify mechanisms responsible for drug activity in order to improve the effectiveness of anticancer therapy. In our study we examined the extent of DNA damage and the efficiency of its repair in head and neck cancer cells after genotoxic treatment with increasing concentrations of cisplatin,
up to 10 μM, and γ-radiation up to 50 Gy. We also examined the extent of DNA damage after hydrogen peroxide treatment within the concentration range 2.5 to 50 μM. The time course of repair of DNA damage was examined at five points during 240 min of post-treatment incubation repair.

In previous studies, the comet assay has been used to examine the association of DNA repair capacity in peripheral blood lymphocytes with the risk of HNSCC (Parshad and Sanford, 2001; Hoeijmakers, 2001; Iwakawa et al., 2005; Saha et al., 2008). The studies showed an increased level of DNA damage and decreased repair in HNSCC patients. The authors also reported high variability in background DNA damage, which might suggest the genetic instabilities in HNSCC cells. Palyvoda et al. (2002) showed decreased repair in HNSCC patients which in part might explain the increased baseline of DNA damage. These results suggest that head and neck cancer cells are highly sensitive to DNA damage, thus treatment with DNA-reactive drugs and radiation might be considered as an effective therapy strategy. However, the data are still confusing especially in advantage stage of this disease (Sotiriou et al., 2004), and the molecular basis for successful therapy of HNSCC patients remains unclear. The monitoring of DNA damage and its repair should be analyzed on tar-

Fig. 4. Time course of the repair of DNA damage in (A) lymphocytes from healthy donors, (B) lymphocytes from HNSCC patients, (C) HTB-43 larynx cancer cells, (D) cells from tissue of healthy donors, (E) cells from tissue of HNSCC patients, and (F) cells from tissue of patients with metastasis irradiated at dose of 15 Gy (—) or 25 Gy (—) compared with untreated controls (——). The number of cells in each treatment was 100. Error bars denote SEM.
get tissue. The assumption is that the DNA repair capacity of an individual is a genetic predisposition measurable in various cell types. In order to increase the reliability, besides peripheral blood lymphocytes we used tissue samples from biopsies in our study.

In our work all types of analyzed cells were sensitive to genotoxic treatment resulting in a decrease of their viability. However, we observed significant differences in cell viability, measured by the MTT assay, between cells from HNSCC patients or HTB-43 cells and healthy donors cells. It was found that lymphocytes from patients were more sensitive to genotoxic treatment with hydrogen peroxide, cisplatin and γ-radiation. After γ-radiation treatment it was observed a higher sensitivity to DNA damages in cells from HNSCC patients than healthy controls measured as percentage of DNA in the comet tail. While a significantly higher level of DNA damage was observed in HNSCC lymphocytes than healthy lymphocytes after cisplatin treatment, we did not find any differences of DNA damages between cells from tissue biopsies. Finally, we estimated a slower kinetic of DNA repair after hydrogen peroxide and γ-radiation treatment in lymphocytes and tissue samples from HNSCC patients as compared to healthy controls. A deeply defective repair was found in cells from tissue biopsies of patients with metastasis. The HTB-43 larynx cancer cell line also showed low efficiency of DNA repair after hydrogen peroxide and γ-radiation treatment. While cisplatin generated cross-links, we found that lymphocytes and tissue cells from patients as well as healthy controls were able to completely recover after treatment with cisplatin, and only cells from HNSCC metastasis showed an incomplete DNA repair. Since cell exposure to hydrogen peroxide causes base-free radical modifications (Seeberg et al., 1995; Lindahl, 2001; Caldecott, 2001; López-Lázaro, 2007) and γ-radiation results mainly in strand breaks (Parshad and Sanford, 2001; Lawrance et al., 2003), we suggest that genotoxic treatment may affect mostly BER and NHEJ and/or HRR pathways in HNSCC cells.

In our study we have reported that differences between HNSCC and healthy cells’ sensitivity to DNA damage may be an important issue of successful anticancer therapy. Interestingly among all types of trials performed in our study we could observe that cells treated with cisplatin had the highest efficiency of DNA repair. On the other hand, the factor that decreased this process the most was γ-radiation. Our data suggest, that the molecular basis for this finding may be the less effective DNA repair in HNSCC cells. Finally, we have also evidenced impaired DNA repair in advanced-stage disease with distinct metastasis. In conclusion, we suggest that the genotoxic sensitivity of HNSCC cells may depend on their DNA repair capacity what in turn may be connected with the different effectiveness of early- and advantage stage of head and neck cancer therapy.

Acknowledgements

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by high throughput alkaline comet assays in EBV-transformed cell lines and peripheral blood cells from cancer patients and healthy volunteers. Mutat. Res. **588**, 1–6.


