Apoptosis and cell surface GRP78 expression in benign and malignant parotid gland tumors

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Abstract

Introduction
Glucose-regulated protein 78 (GRP78) has emerged as an important regulator of tumour cell signalling and viability. We have analysed cell surface GRP78 in benign and malignant parotid salivary tumours in correlation to apoptosis.

Materials and methods
Primary cell-cultures were produced from tissue biopsies. Cell surface GRP78 stained by anti GRP78 antibody was analysed by fluorocytometry. Apoptosis was determined by Annexin V/PI.

Results
A significant decrease in the percentage of cell surface GRP78 was determined in the benign parotid tumour in comparison to normal tissue. Cell surface GRP78 expression in malignant tumours was significantly higher and correlated with decrease in apoptosis.

Conclusion
This study is the first report describing cell surface GRP78 expression in parotid salivary tumour. The evidence of high surface GRP78 expression in malignant parotid tissue in contrast to the benign tumour might add a diagnostic tool and serve as a future target for treatment of malignant parotid tumours.

Introduction
Salivary gland neoplasm, commonly located in the parotid gland, constitutes about 3-5% of all head and neck tumours. The most common neoplasm of the salivary glands is benign pleomorphic adenoma originating from the parotid gland. Malignant tumours of the salivary glands consist of only 10% of the parotid tumours. The most common malignant salivary gland tumour is mucoepidermoid carcinoma followed by adenoid cystic carcinoma and ex pleomorphic carcinoma.

The aetiology of salivary gland neoplasm is not known; however, it has been associated with environmental factors such as Epstein-Barr virus (EBV) infection, exposure to ionizing radiation, smoking and alcohol consumption. Additionally, an increasing pool of evidence has demonstrated the role of various genetic alterations in the formation of salivary neoplasms. The overall outcome of patients with malignant salivary cancer is unfavourable. Therefore, researchers are seeking for promising new therapeutic modalities to improve traditional therapy. In general, potential targets are molecular signalling pathway factors, tumour target receptors and antibodies. Recently, it was suggested that glucose-regulated protein 78 (GRP78), a member of the heat shock protein 70 family (Hsp70), found to be over-expressed on the cell surface of tumour cells, might be used as a new therapeutic target.

Basically, GRP78 is a central regulator of the unfolded protein response (UPR) in the endoplasmic reticulum (ER) due to its role in nascent protein quality control in which survival or apoptotic pathways are activated. The intracellular induction of GRP78 might lead under stress conditions to its re-localization to the cell surface where it assumes a new function as a receptor for signalling. Cell surface GRP78 has emerged as an important regulator of tumour cell signalling and viability as it forms complexes with a repertoire of cell-surface protein partners. In certain tumours the increased tumorigenicity has been attributed to the over expression of GRP78.

Although the expression of Hsp70 in a human parotid salivary gland was demonstrated, the presence of cell surface GRP78 in these cells has not been described. In this study we analysed cell surface GRP78 expression in benign and malignant parotid salivary cells and correlated the level of cell surface GRP78 expression on benign and malignant tumour cells to apoptosis.

Materials and methods

Patients
The study was conducted on fresh tissue samples from patients who were operated in the department of Otorhinolaryngology head and neck surgery at the Rabin Medical Center and was approved by the Institutional Helsinki committee.

The study cohort consisted of 20 patients. There were 14 males and 6 females. The mean age at diagnosis was 42.7 years, with a range from 18 to 85 years.

Competing interests: None declared.

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The most common presenting symptom for patients with benign tumour of the parotid gland was a slowly growing, painless upper neck mass. The mean duration of symptoms before diagnosis was 34.5 months (range 18-48).

Conversely, the most common symptom in patients with malignant disease was a fast growing parotid mass with local pain. Two patients with malignancy demonstrated enlarged neck nodes suspicious for metastases. None of the patients presented with facial nerve palsy. The pre-operative evaluation included an ultrasonographic examination and a fine needle aspiration of the tumour. The fine needle aspiration differentiated between benign and malignant disease in all patients. In all cases, the tumour was located in the parotid gland.

Patients with a benign disease underwent superficial parotidectomy, whereas patients with malignant disease underwent a neck dissection as well. Normal parotid tissue was collected from 11 patients during parotidectomy for benign lesions or from the tail of the parotid during neck dissections for causes unrelated to cancer.

**Primary cell culture**

Fresh tissue was transferred directly from the operating room to the laboratory. A short time after removal, the tissue was minced into pieces smaller than 1 mm and washed three times with PBS and centrifuged at 1200 rpm for 12 min. The pellet was suspended in DMEM medium with 10% foetal bovine serum supplemented with 2% glutamine, 1% pyruvate and antibiotic solution (penicillin, streptomycin and nistatin, Biological Industries, Kibbutz Beit HaEmek, Israel). The minced tissue was then dispersed in 60mm Petri dish plates and incubated at 37°C for 24 h in a 5% CO2 incubator. The medium was changed every 48 h until Petri dishes plastic- attached epithelial cells reached confluence growth. Some cultures were subjected to hypoxic conditions for 24 hours before assay using a gas mix of 94% nitrogen + 5% CO2 + 1% O2 in a hypoxia chamber (Billups- Rothenberg, San Diego, CA, USA).

Cultures were stained with Haematoxylin -Eosin. Mayer’s Haematoxilin was added for 3 minutes and immediately washed with tap water. Eosin 1% aqueous, diluted 1:10 was added for 1 minute and washed with tap water for 3 minutes (Pioneer Research Chemicals Limited, Colchester Essex CO2 8HX, UK). Photomicrographs were taken from a microscope (Olympus 51BX) at 10X magnification (Bars=0.2mm).

**Analysis of cell surface GRP78**

Cells were removed for fluorocytometric analysis by incubation with trypsin-EDTA (0.025%-0.05%) (Biological Industries, Beit Ha Hemeck, Israel) and single cell suspension in PBS supplemented with 5%FBS and 0.01% Sodium Azide. Fluorescent staining was proceeded by incubation with anti GRP78 polyclonal antibody (Santa Cruz, Biotechnologies, CA, USA) for 45 minutes followed by anti-goat FITC for 30 minutes at 4°C. Isotype control for non-specific binding was determined by goat IgG antibody followed by anti-goat FITC. Percentage of GRP78 positive cells were analysed by the fluorescence cell sorter (FACScan Beckton Dickinson, San Jose, CA, USA). Results are expressed as mean of the percentage of membrane positive cells ±SE in 3 different groups.

**Apoptosis assay**

Apoptosis was determined by staining the harvested single cell suspensions with Annexin V-FITC kit (Becto Apoptosis kit, MBL, Naka-Ku Nagoya, Japan) following the manufacturer’s instructions. Cells were analysed by the fluorescence activated cell sorter (FACScan Beckton Dickinson, San Jose, CA, USA). The percentage of Annexin-V-positive and PI-negative cells were scored as early apoptosis and the percentage of Annexin-V-positive and PI

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*Figure 1: H&E staining of primary cultures of human parotid cells. Representative microscope photographs of one week in culture of plated cells from normal, benign and malignant tumors. Cells were stained with Hamatoxylin & Eosin. Pictures were taken at 10X magnification (Bars=0.2mm) from Olympus 51BX microscope.*

*Figure 2: FACS analysis of cell surface GRP78 expression. Upper row: Representative dot-plot from normal, benign tumor and malignant tumor cells population showing size and granulation of cells. Lower row: Representative histograms showing the percent of GRP78 positive cells stained by goat anti GRP78 antibody (red line) analyzed by fluorescence cell sorter. The isotype control (black line) for non specific staining was determined by staining with goat IgG antibody followed by anti-goat FITC.*

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positive cells were scored as late apoptosis. Results are expressed as percentage of apoptosis ±SD.

Statistical Analysis
A statistical analysis was carried out using the JMP software (SAS Institute, Cary, NC, USA). Data were expressed as mean and standard deviation (SD) or standard error (SE). Differences between in the different groups were calculated by One way analysis of variance (ANOVA) for multiple comparisons. P-values below 0.05 for all tests were considered statistically significant.

Results
Histopathological data
The final pathology confirmed pleomorphic adenoma in 6 cases, Acinic cell carcinoma (1 patient) and high grade Mucoepidermoid carcinoma (2 patients). The mean maximal diameter for the pleomorphic adenomas was 2.18 cm (range 1.5-3 cm). The mean maximal diameter of the malignant tumours was 1.73 (ranging from 12.2-2.2 cm). Histological examination confirmed the presence of regional disease (4/7 and 10/32 metastatic nodes out of total nodes excised respectively) in the 2 patients with mucoepidermoid carcinoma who presented with suspicious neck nodes at presentation. Both patients received post-operative radiation. None of the patients had a distant metastasis. At last follow up, the patient with acinic cell carcinoma had no evidence of disease. The two patients who were diagnosed with mucoepidermoid carcinoma experienced recurrence and eventually died of their disease. Patient’s clinical data is summarized in table 1.

Primary cell cultures from normal, benign and malignant tumours
Cultures obtained from normal parotid biopsies were found to be difficult to grow since the cells did not adhere to plates easily. However, after 2-3 weeks in culture a sufficient number of cells enabled the different experimental assays. In contrast, benign and malignant tumours grew rapidly and adhered strongly to the Petri dishes. Cell monolayer was visible after 1-2 week incubation. Cells cultures from all three groups were stained after two weeks in culture by H&E incubation. Cells cultures from all three groups were cultured from normal parotid biopsies were fluorescently stained for cell surface GRP78 and the percentage of positive surface GRP78 cells in normal, benign and malignant parotid cells under normoxic and hypoxic conditions. Summary of results of multiple fluorocytometric analyses of percent surface GRP78 positive cells isolated from normal, benign and malignant tumor cells under normoxic conditions or exposed to 24h of hypoxic conditions prior to analysis. A significant (p<0.05) 2.2 fold decrease in the percent of GRP78 in cell surface of benign tumor in comparison to normal tissue was observed both under normoxic or hypoxic conditions. In contrast, the percent of positive surface GRP78 cells in malignant tumors demonstrated a significant increase (p<0.05) of 1.8 fold compared to normal cells and a 4 fold increase over positive cells in the benign tumors. No significant difference was seen in percent of cells under hypoxic conditions. Results are expressed as Mean percent ±SE.

Analysis of cell surface GRP78 under normoxic and hypoxic conditions.
Live cells obtained from primary cultured benign, malignant and normal parotid biopsies were fluorescently stained for cell surface GRP78 and the percentage of receptor positive cells was determined by FACS analysis. Representative dot plots of normal, benign tumour and malignant tumour in hypoxic conditions prior to analysis. A significant (p<0.05) 2.2 fold decrease in the percent of GRP78 in cell surface of benign tumor in comparison to normal tissue was observed both under normoxic or hypoxic conditions. In contrast, the percent of positive surface GRP78 cells in malignant tumors demonstrated a significant increase (p<0.05) of 1.8 fold compared to normal cells and a 4 fold increase over positive cells in the benign tumors. No significant difference was seen in percent of cells under hypoxic conditions. Results are expressed as Mean percent ±SE.

Figure 3: Determination of percent cell surface GRP78 positive cells in normal, benign and malignant parotid cells under normoxic and hypoxic conditions. Summary of results of multiple fluorocytometric analyses of percent surface GRP78 positive cells isolated from normal, benign and malignant tumor cells under normoxic conditions or exposed to 24h of hypoxic conditions prior to analysis. A significant (p<0.05) 2.2 fold decrease in the percent of GRP78 in cell surface of benign tumor in comparison to normal tissue was observed both under normoxic or hypoxic conditions. In contrast, the percent of positive surface GRP78 cells in malignant tumors demonstrated a significant increase (p<0.05) of 1.8 fold compared to normal cells and a 4 fold increase over positive cells in the benign tumors. No significant difference was seen in percent of cells under hypoxic conditions. Results are expressed as Mean percent ±SE.

Figure 4: Determination of early and late apoptosis in normal, benign tumour and malignant tumor. Apoptosis was determined by staining the cells originating from normal parotid, benign and malignant tumors with Annexin-V-FITC and propidium iodide (PI), followed by fluorescence activated cell sorter (FACS) analysis. Parallel cultures were exposed to 24 hours hypoxia prior to cell harvesting. The percent of Annexin-V-positive and PI-negative cells were scored as cells in early apoptosis and the percent of Annexin-V-positive and PI positive cells were scored as cells under late apoptosis. As can be seen in summary of results obtained by repeated experiments, cells derived from malignant tumors showed a highly significant (p<0.05) decrease in both early and late apoptosis. Percent of apoptosis, both early and late, in the benign tumor cells did not differ from that of normal cells. Results are expressed as percent of apoptotic cells ±SD.

Figure 3: Determination of percent cell surface GRP78 positive cells in normal, benign and malignant parotid cells under normoxic and hypoxic conditions. Summary of results of multiple fluorocytometric analyses of percent surface GRP78 positive cells isolated from normal, benign and malignant tumor cells under normoxic conditions or exposed to 24h of hypoxic conditions prior to analysis. A significant (p<0.05) 2.2 fold decrease in the percent of GRP78 in cell surface of benign tumor in comparison to normal tissue was observed both under normoxic or hypoxic conditions. In contrast, the percent of positive surface GRP78 cells in malignant tumors demonstrated a significant increase (p<0.05) of 1.8 fold compared to normal cells and a 4 fold increase over positive cells in the benign tumors. No significant difference was seen in percent of cells under hypoxic conditions. Results are expressed as Mean percent ±SE.

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malignant tumour cells population is presented in figure 2 upper row that shows similarity in size and granulation of the cell populations. A representative FACS analysis that determines the percentage of cells expressing cell surface GRP78 in each tumour group is presented in figure 2 lower row. As can be seen, the Isotype control (black line) determined the background control of non-specific binding while the red line demonstrates the positive staining. In the malignant group there is an increase of percentage positive stained cells above the non-tumour control cells. We therefore assumed that incubating the cells under hypoxic conditions will induce a significant increase (11.16±2.96 in hypoxia). In contrast, malignant tumour cells contained a significant increase to 50±10.4 percent of GRP78 receptor positive cells, which is 1.8 times higher than control cells and 4 fold higher than cells derived from benign tumours. Cell surface GRP78 in the malignant tumour was 35.2±11 under hypoxic conditions.

Apoptosis of isolated cell from normal, benign and malignant tumours

To analyse the apoptotic pattern in the derived cell of the 3 different tissues, we studied the cell cultures under hypoxic conditions. The results of 3 repeated experiments are presented in figure 4. The percentage of AnnexinV positive cells (early apoptosis) derived from normal and benign tumour were similar, 29.4±1.8 and 28.9±1.8 respectively. The same pattern was observed in late apoptosis where the percentage of AnnexinV/PI positive cells (late apoptosis) derived from the normal and benign tumour were similar, 29.4±1.8 and 28.9±1.8 respectively. The same pattern was observed in late apoptosis where the percentage of AnnexinV/PI positive cells was 11.75±0.1 and 15.2±0.01 in normal and benign tumours. Cells derived from malignant tumours showed a decrease in the early (17.21±1.36) and late apoptosis (4.16±0.1), demonstrating that the cells are more resistant to hypoxia induced apoptosis.

Discussion

In this study, we assessed cell surface GRP78 expression for the first time in benign and malignant salivary gland neoplasm originating in the parotid gland. Benign tumours were removed from six patients with pleomorphic adenoma. Malignant tumours were obtained from one patient with acinic cell carcinoma and two with high grade Mucoepidermoid carcinoma. Primary Cell cultures from fresh parotid samples transferred directly from the operating room to the laboratory is presented in this study. The results obtained demonstrated a 4 fold increase in malignant tumour cell surface GRP78 compared to benign tumours. It is important to note that despite the different origin of the malignant tumour cells, the percentage of cell surface GRP78 was similar in all the malignant tumour cell samples. The absence of most cell surface GRP78 in benign tumours was significant, being 2

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fold less than in non tumour parotid cells. The data also demonstrated no induction of cell surface GRP78 in the parotid tumour cells under hypoxic conditions. Hypoxic conditions were found to induce cell surface GRP78 in certain cell types such as endothelial cells, cardiomyocytes, and neurons. Several tumour cell types manifest increased cell surface GRP78 such as in breast cancer cells, neuroblastoma glioma hybrid cell line, lung adenocarcinoma, colon adenocarcinoma and others. The presence of cell surface GRP78 on tumour cells was attributed to the deficiencies in the tumour microenvironment such as lack of oxygen and nutrients. A correlation between high expression of GRP78 and aggressive tumours was suggested. The low cell surface GRP78 in benign parotid tumours compared to the malignant cells follows this concept. However, in our previous study, we analysed the tumorigenic activity of GRP78 positive and negative cells within two colon carcinoma cell lines and found that GRP78 receptor negative sub-population represented tumorigenicity and metastatic activity. The conflicting outcome might result from the different origin of the tumours.

Most of the tumours in the parotid gland are benign and only a small percentage are malignant with metastatic potential. Other than Ex-pleomorphic adenocarcinoma that was not included in the current study, benign tumours of the parotid gland do not represent an early stage of malignant development, as opposed to colon carcinoma. Malignant and benign parotid tumours originate from distinct origins.

It was previously demonstrated that surface GRP78 functions as a receptor involved in the regulation of cell growth and apoptosis. Additionally, it was shown that over-expression of GRP78 in aggressive tumours renders these cells resistant to chemotherapy drugs induced apoptosis. These observations support the results obtained in our study in which parotid cell cultures were exposed to 24 hours hypoxia prior to the determination of apoptosis. Cells derived from the malignant tumours showed a significant decrease in both early and late apoptosis. Hypoxia in such conditions induced principally early apoptosis; however malignant tumour cells showed resistance to early and late apoptosis. The percentage of apoptosis, both early and late, in the benign tumour cells did not differ from normal cells.

**Conclusion**

This study amplifies the value of targeting GRP78 to race tumorigen-esis and resistance to chemotherapy in parotid gland malignant tumours. This study is the first report describing cell surface GRP78 expression in parotid salivary tumour.

The evidence of high surface GRP78 expression in malignant parotid tissue in contrast to the benign tumour might add a diagnostic tool and serve as a future target for treatment of malignant parotid tumours.

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