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The expressions of intrinsic and extrinsic apoptotic pathway proteins in neutrophils of oral cavity cancer patients: a preliminary study

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Abstract

Introduction: The biological availability and activity of polymorphonuclear neutrophils (PMNs) are regulated by their short life span, which can be additionally shortened by a malignant process. The signaling pathways leading to apoptotic PMN death are classified in two categories: the intrinsic and the extrinsic. In the present study the expressions of proteins participating in the extrinsic apoptotic pathway (DR5, FADD, caspase-8 activity) and the intensity of apoptosis of PMNs from patients with cancer of the oral cavity were examined. The expression of proteins participating in the intrinsic pathway (Bax and Mcl-1) were also examined in these cells. The results can be helpful in explaining the reasons for the decreased activity of these cells in oral cavity cancer patients.
Materials and Methods: The examinations were carried out in patients with squamous cell carcinoma of the oral cavity before and after treatment. The expressions of all the proteins were measured in neutrophils and, for comparison, in autologous peripheral blood mononuclear cells (PBMCs). Western blot analysis was used to assay the expressions of DR5, FADD, Bax, and Mcl-1 in cell lysates. The apoptosis level was determined by flow cytometry and caspase-8 activity by colorimetric assay.
Results: A lack of changes in DR5 expression associated with increased FADD protein expression and caspase-8 activity accompanied the accelerated apoptosis rates in the PMNs of the patients before treatment. Decreased expression of anti-apoptotic Mcl-1 protein was associated with an unchanged expression of pro-apoptotic Bax protein. There were no such changes in the patients PBMCs. Increased expression of Mcl-1 in the PMNs of the patients following surgical treatment was found.
Conclusion: The acceleration of the apoptosis of PMNs of oral cavity cancer patients before treatment is dependent on both the intrinsic and extrinsic pathways.

Key words: polymorphonuclear neutrophils, mononuclear cells, apoptosis, oral cavity carcinoma.

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INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are natural effector cells mediating antibacterial, antifungal, and antitumor defense (Di Carlo et al. 2001; Koga et al. 2004; Stoppacciaro et al. 1993). Their biological availability and activity are regulated by their short life span, which can be additionally shortened by a malignant process (Maianski et al. 2004). Signaling pathways leading to the death of neutrophils along the apoptotic pathway are classified into two categories: the intrinsic and the extrinsic, both of which have the same outcome: the activation of a characteristic caspase cascade (Simon 2003; Sprick and Walczak 2004). The intrinsic pathway is activated by mitochondrial disruption with subsequent cytochrome c release, Smac/DIABLO, and Omi/HtrA2. This pathway is regulated by members of the Bcl-2 family of proteins (Simon 2003). Neutrophils constitutively express Bcl-2 family members such as the pro-apoptotic Bax and anti-apoptotic Mcl-1 (Moudling et al. 1998). The extrinsic pathway involves the binding of ligands to cell-surface "death receptors", which in turn initiates the caspase cascade. Death receptors belong to the tumor necrosis factor (TNF) superfamily of proteins

and provide a rapid and efficient route to apoptosis. In different situations, neutrophils are sensitive to the proapoptotic stimuli FasL, TNF, and TNF-related apoptosis-inducing ligand (TRAIL) (Simon 2003).

TRAIL exerts its apoptotic activity by interacting with a complex system of two death receptors, TRAIL--R1(DR4) and TRAIL-R2 (DR5) (Almasan and Ashkenazi 2003). It has been demonstrated that human neutrophils express both TRAIL-R2 mRNA and protein, which is capable of transducing a pro-apoptotic signal by activating caspase-8 through the recruitment of an adaptor protein, Fas-associated protein with death domain (FADD) (Wang and El-Deiry 2003). The presence of TRAIL and TRAIL receptors in neutrophils suggests that this complex might play a role in their apoptosis (Kamohara et al 2004). Our previous study on neutrophils of patients with oral cavity cancer showed a low expression and secretion of TRAIL molecule, without changes in the expression and secretion of TRAIL-R2 receptor (DR5), which makes them sensitive to TRAIL secreted by other cells (Jablonska et al. 2008).

To explain whether the preservation of the level of death receptor DR5 is involved in apoptotic signaling in the PMNs of patients with oral cavity cancer, we compared its expression with FADD expression and caspase-8 activity and with the intensity of apoptosis of these cells. Our aim in this study was also to examine the potential role of the intrinsic apoptotic pathway with the participation of Bax and Mcl-1 proteins in the survival of the examined neutrophils. The results can be helpful in explaining the reasons for the decreased activity of these cells in oral cavity cancer patients (Jablonska et al. 2005).

MATERIALS AND METHODS

Patients

Twenty patients aged 45–49 years with squamous cell carcinoma of the oral cavity treated at the Department of Oral and Maxillofacial Surgery, Medical University of Bialystok, were examined. The examinations were carried out on the patients before treatment and three weeks after the surgical removal of the tumor mass. The patients did not receive any treatment or medication before the examination. The results were analyzed according to TNM staging (Sobin and Wittekind 1997). There were no clinical signs of infection observed in the patients. The patients had no significantly increased leukocytosis. The control subjects (n=15) were healthy people aged 30–53 years (mean: 41.5 years). The study was approved by the Ethics Committee of the Medical University of Białystok and all the patients submitted their consent in writing.

Preparation of PMNs and PBMCs

For the purpose of comparison, the expressions of all the examined proteins were also determined in autologous peripheral blood mononuclear cells (PBMCs). The cells were isolated from whole blood treated with EDTA by density centrifugation using Polymorphoprep (Axis-Shield, Oslo, Norway) (density: 1.113 g/ml). This method enables the simultaneous separation of two highly purified leukocyte fractions: mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs), involving neutrophils. The purity of the isolated PMNs and PBMCs was determined by May-Grunewald-Giemsa--staining.

Western blot analysis

The cytoplasmic protein fractions of the PMNs and PBMCs were analyzed by Western blotting for the presence of DR5, FADD, Bax, and Mcl-1. The cells were lysed directly in the presence of Protease Inhibitor Cocktail (Sigma-Aldrich, CHEMIE GmbH P.O. Steinheim, Germany) by sonication using a Vibra-Cell Ultrasonic Processor (Sonics&Materials, Inc., USA). The protein fractions were suspended in Laemmli buffer (Bio-Rad Laboratories, Herkules CA, USA) and then electrophoresed on SDS-PAGE. The resolved protein was transferred onto 0.2-µm pore-sized nitrocellulose (Bio-Rad Laboratories, Hercules CA, USA). The nitrocellulose was incubated at 4°C for 20 h with anti--DR5 anti-FADD primary polyclonal antibody and monoclonal anti-Bax (R&D Systems) and anti-Mcl-1 (Biosource). After washing in 0.1% TBS-T, the membrane was incubated at room temperature for 1 h with alkaline phosphatase anti-mouse IgG Abs (Vector Laboratories, Burlingame, CA, USA). The immunoreactive protein bands were visualized following the addition of AP-Conjugate Substrate Kit (Bio-Rad Laboratories, Herkules CA, USA). The bands' intensities were quantified using LabImage 1 D gel software.

Apoptosis evaluation

Annexin V binding of neutrophils was performed using an apoptosis detection kit (APOTEST^{TM} – FITC, Nexins Research, Holland). Initially, 10^5 cells/ml of freshly isolated cells or incubated cells were labeled with FITC-conjugated Annexin V and propidium iodide (PI) for 20 min at 4°C. The samples were analyzed by two--color flow cytometry using CellQest Analysis software (Becton Dickinson, Epics Coulter, USA). The results were reported in the form of the percentage of Annexin V-positive cells, which reflects the relative proportion of apoptotic cells. Cells positive for PI and Annexin V--FITC and PI were considered necrotic.

Caspase-8 activity

Caspase-8 activity was measured using a Caspase-8 Colorimetric Assay (R&D Systems). The cell lysates were tested for protease activity by the addition of a caspase-specific peptide conjugated to the color reporter molecule p-nitroaniline (pNA). The cleavage of the

peptide by the caspase releases the chromophore pNA, which was quantified spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction.

Statistical analysis

Statistical analysis was performed with the statistics package Statistica 6.0 (Statsoft, Kraków, Poland). The non-parametric Mann-Whitney U-test was used. Results were expressed as the median and the maximum and minimum values. p values smaller than 0.02 were considered statistically significant.

RESULTS

Western blot analysis

Western blot analysis showed no difference in the expressions of DR5, a 58-kDa protein, in the PMN and PBMC lysates of the control and patient groups (Fig. 1). Its expression in the PMNs of the patients was lower than in autologous PBMCs. DR5 expression in the PMN and PBMC lysates was on the same level in the patients before and after treatment.

The expression of FADD, a 21-kDa protein, in the PMNs of the patients before treatment was higher than in the control cells (Fig. 1). After treatment, the expres-

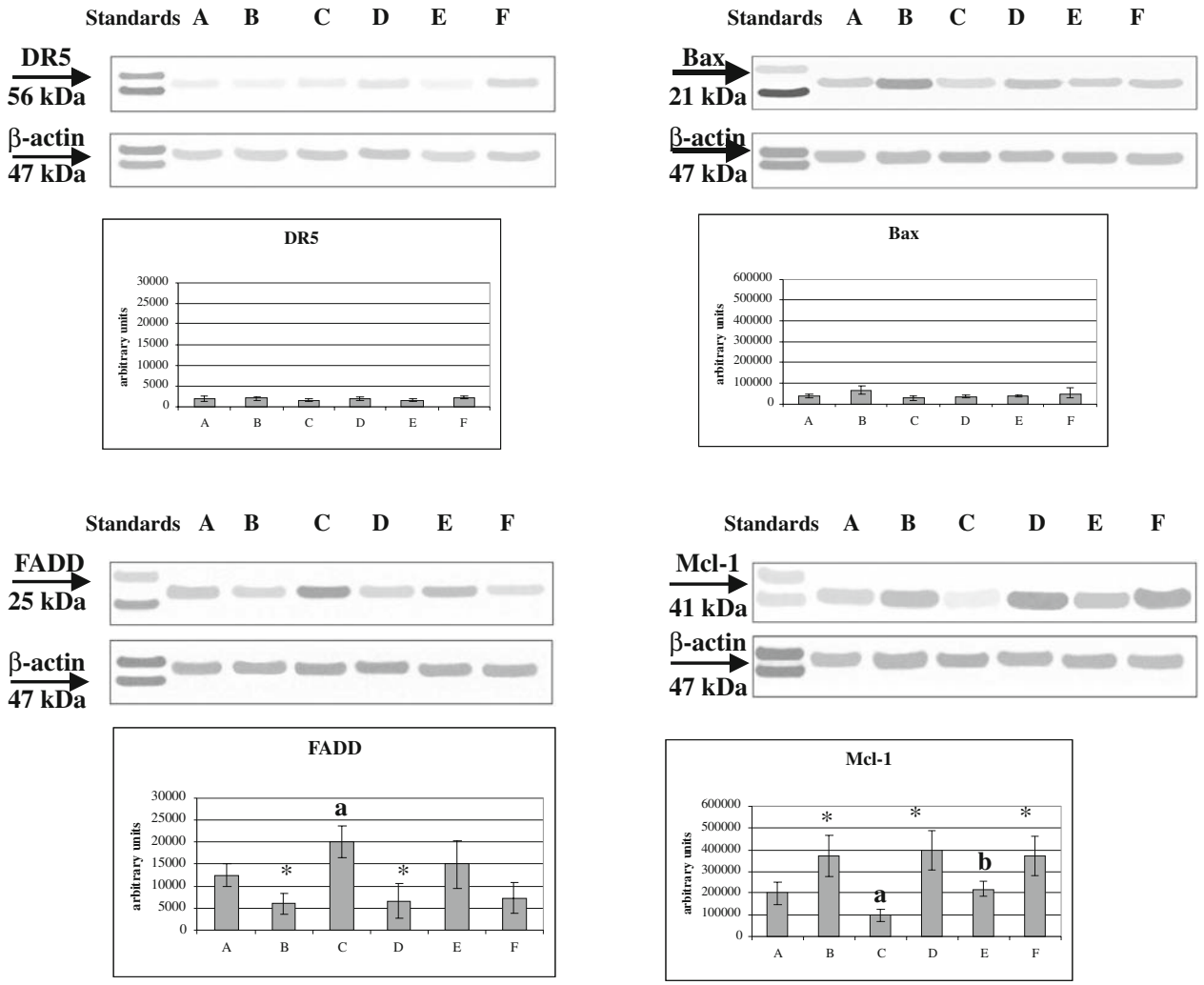


Fig. 1. Western blot analysis of DR5, FADD, Bax, and Mcl-1 protein expression in PMNs and PBMCs of control and oral cavity cancer patients: **A** – PMNs of controls, **B** – PBMCs of controls, **C** – PMNs of patients before treatment, **D** – PBMCs of patients before treatment, **E** – PMNs of patients after treatment, **F** – PBMCs of patients after treatment. The whole-cell lysates were obtained from fresh PMNs. One hundred micrograms of total protein from the cells were loaded onto a 10% SDS-PAGE and transferred to a nitrocellulose filter. The filter was incubated with anti-DR5 anti-FADD polyclonal antibody and anti-Bax and Mcl-1 monoclonal antibody. The diagram below shows the statistical analysis of the Western blots of DR5, FADD, Bax, and Mcl-1 from seven independent experiments. Band intensity was quantified using LabImage 1 D gel software and presented in arbitrary units. *Statistically significant differences between PMNs and PBMCs ($p<0.05$). [a]Statistically significant differences between patients and control ($p<0.05$). [b]Statistically significant differences between patients before and after treatment ($p<0.05$).

sion was on the same level. FADD expression in the PMNs of the patients and controls was higher than in their PBMCs.

There were no differences in Bax expression, a 21--kDa protein, in the PMN and PBMC lysates of the patients before treatment and the controls (Fig. 1). Furthermore, Bax expression in the PMNs of the patients after treatment was on the same level as before treatment.

The expression of Mcl-1, a 41-kDa protein, in the PMNs of the patients before treatment was lower than in the controls (Fig. 1). After treatment, increased expression of Mcl-1 in the PMNs was observed. Mcl-1 expression in the PMN lysates of the patients and controls was lower than in PBMCs.

Apoptosis

It was observed that 0.64% of the freshly isolated PMNs of the controls had features of apoptosis (Annexin V-binding cells), while 1.2% showed features of necrosis (PI-binding as well as PI- and Annexin V--binding cells) (Fig. 2). In the patients before treatment the percentage of apoptotic PMN was 12.6% and of necrotic cells 3.8%. After treatment, the percentage of apoptotic PMNs decreased to 5.8% and necrotic cells to 1.8% (Fig. 2). In contrast, the number of autologous PBMCs with features of apoptosis in the patients before and after treatment were on the level of apoptosis as the cells of the control group (0.91, 1.6 and 0.73, 1.2%, respectively).

Caspase-8 activity

Caspase-8 activity in the PMN lysates from patients before treatment was higher than in the cells of the control group and lower than in the patients after treatment (Table 1). The activity of this enzyme in the autologous PBMC lysates of the patients was on the control group level.

DISCUSSION

In the this report we presented an interesting relationship among molecules participating in the extrinsic apoptotic pathway and the intensity of apoptosis of PMNs in oral cavity cancer patients before treatment. The absence of changes in DR5 expression, associated with the increased expression of FADD protein and caspase-8 activity, accompanied accelerated apoptosis rates of these cells. These results suggest that the extrinsic pathway may be responsible for the shortened life span of PMNs in this patient group, but on a pathway other than that mediated by TRAIL. Since human neutrophils are susceptible to FasL- and TNF-induced apoptosis, it is possible that these ligands may result in an increased intensity of apoptosis of PMNs of oral cavity cancer patients (Simon 2003). Gastman et al. demonstrated that Fas ligand is expressed on human squamous cell carcinomas of the head and neck and that it promotes apoptosis of T lymphocytes (Gastman et al. 1999).

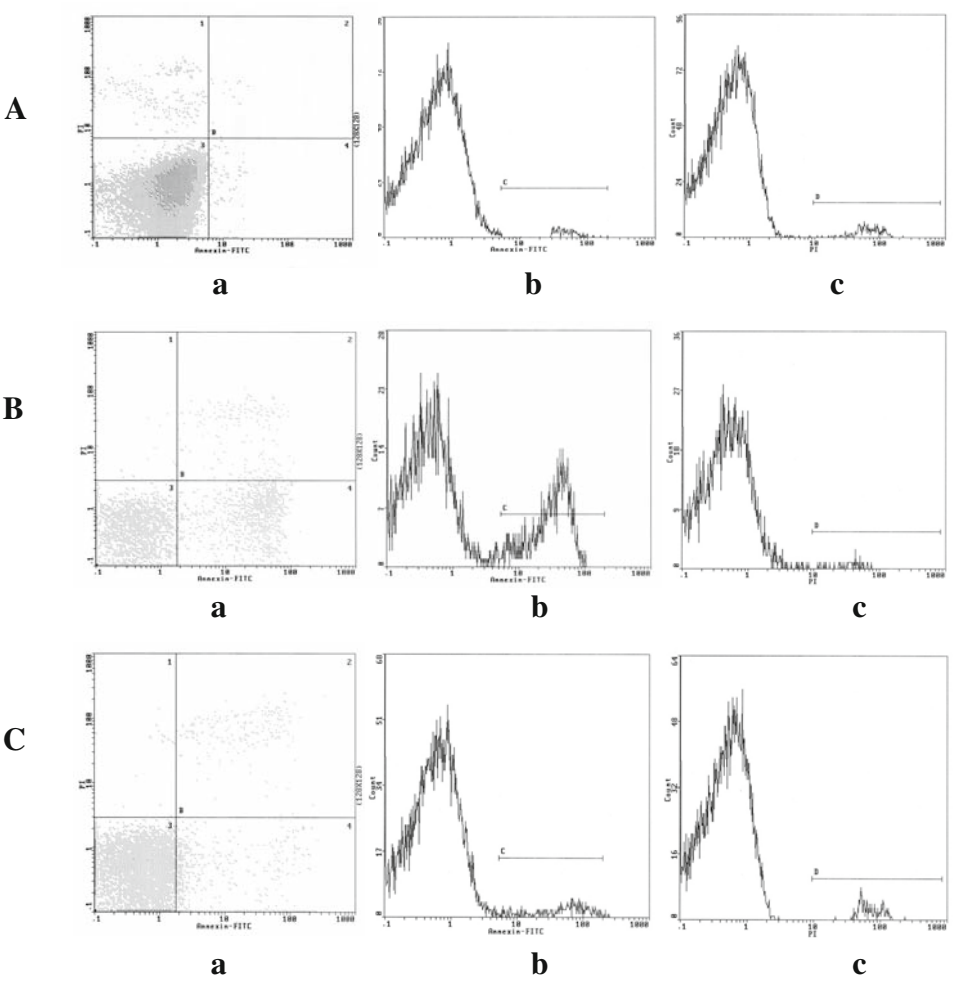
The enhanced apoptosis of PMNs in oral cavity cancer patients may also be attributable to a relationship between the expressions of Bcl-2 family proteins. Decreased expression of anti-apoptotic Mcl-1 protein was associated with an unchanged expression of pro-apoptotic Bax protein. The imbalance between Mcl-1 and Bax and the simultaneously enhanced apoptosis of neutrophils suggest that this process may also be influenced by the deficit of Mcl-1 protein. This is in agreement with other reports indicating that cellular levels of Mcl-1 in human neutrophils are associated with increased cell survival and that apoptotic neutrophils have low levels of this protein (Gastman et al. 1999; Simon 2003). It is known that Mcl-1 interacts with tBid and impairs its ability to induce apoptogenic cytochrom c release from mitochondria (Edwards et al. 2004). Recently, Bim protein was identified as a high-affinity partner for Mcl-1, which inhibits Bim from releasing mitochondrial cytochrom c (Clohessy et al. 2006). Thus the loss of Mcl-1 in the PMNs of cancer patients may generate free tBid and Bim, which are involved in the ensuing apoptotic events responsible for the increased intensity of neutrophil apoptosis observed in the present study.

One of the reasons for the decreased expression of Mcl-1 in the PMNs of cancer patients might be the activity of caspase-8, which cleaves Mcl-1, initiating a signal for the TRAIL-mediated mitochondrial cascade (Han et al. 2006). The increased activity of caspase-8 observed in the PMNs of the oral cavity cancer patients appears to confirm its role in the degradation of Mcl-1 in these cells. The increased expression of Mcl-1 in the PMNs of patients after surgical treatment to remove the tumor indicates that degradation of this protein in the patients before treatment was caused by a direct or indirect effect of the tumor cells. The simultaneously delayed

Table 1. The enzymatic activity of caspase-8 in PMNs and PBMCs from patients with oral cavity cancer and the control group

	Caspase-8 activity		
Cells	Control (n=15) median values±min–max	Patients before (n=20) median values±min–max	Patients after (n=18) median values±min–max
PMN	0.142±0.121–0.233	0.219*±0.176–0.292	0.148±0.131–0.231
PBMC	0.149±0.125–0.195	0.174±0.114–0.207	0.163±0.134–0.197

*Significantly different from controls ($p<0.02$).



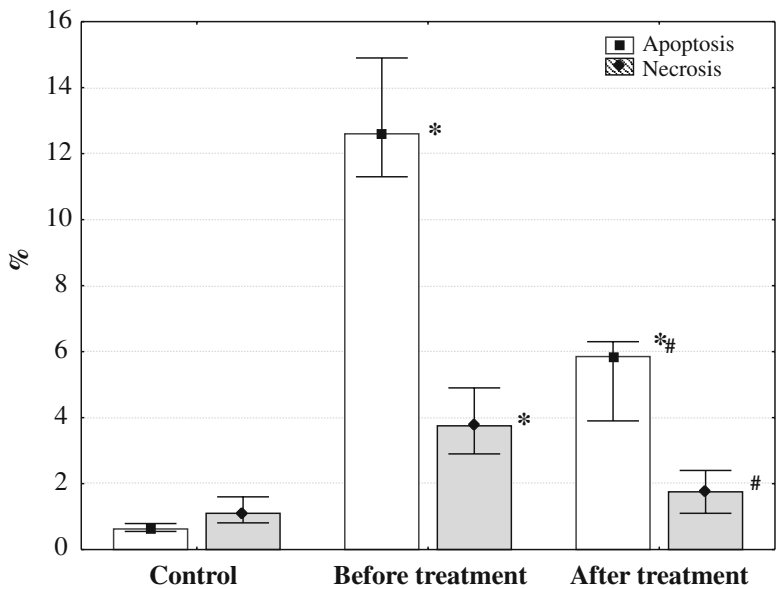


Fig. 2. Study of PMN apoptosis by flow cytometry in the control group (**A**), in the patients before treatment (**B**), and the patients after surgical treatment (**C**). Flow cytometry expression of apoptosis PMN (**a**), PMNs are labeled with Annexin V-FITC (**b**) and propidium iodide (PI) (**c**). Cells stained positive for Annexin V-FITC and negative for PI were considered apoptotic. Cells positive for PI and Annexin V-FITC and PI were considered necrotic. *Statistically significantly different from controls ($p<0.02$). #Statistically significant difference between examinations before and after treatment ($p<0.02$).

apoptosis of these cells confirms a role of anti-apoptotic Mcl-1 protein deficit in the accelerated apoptosis of PMNs in patients before treatment.

In contrast to PMNs, the PBMCs of the patients before treatment exhibited unchanged expressions of FADD and Mcl-1 and caspase-8 activity as well as intensity of apoptosis. These differences suggest that of the examined leukocytes, PMNs are more sensitive to the malignant process than autologous PBMCs.

In conclusion, this preliminary study suggests that the accelerated apoptosis of PMNs from oral cavity cancer patients before treatment is dependent on both the intrinsic and extrinsic pathways. However, further extensive research involving a greater number of proteins playing roles in both pathways is needed to fully understand this process in the neutrophils of oral cavity cancer patients.

REFERENCES

Almasan A, Ashkenazi A (2003) Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. Cytokine Growth Factor Rev 14:337–348

Clohessy JG, Zhuang J, de Boer J et al (2006) Mcl-1 interacts with truncated bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. J Biol Chem 281:5750–5759

Di Carlo E, Forni G, Lollini P et al (2001) The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97:339–345

Edwards SW, Derouet M, Howse M et al (2004) Regulation of neutrophils apoptosis by Mcl-1. Biochem Soc Trans 32:489–492

Gastman BR, Atarshi Y, Reichert TE et al (1999) Fas ligand is expressed on human squamous cell carcinomas of the head and neck, and it promotes apoptosis of T lymphocytes. Cancer Res 59:5356–5364

Han J, Goldstein LA, Gastman BR et al (2006) Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. J Biol Chem 281:10153–10163

Jablonska E, Jablonski J, Marcinczyk M et al (2008) The release of soluble forms of TRAIL and DR5 by neutrophils of oral cavity cancer patients. Folia Histochem Cytobiol 46:177–183

Jablonska E, Puzewska W, Grabowska Z et al (2005) VEGF, IL-18 and NO production by neutrophils and their serum levels in patients with oral cavity cancer. Cytokine 30:93–99

Kamohara H, Matsuyama W, Shimozato O et al (2004) Regulation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and TRAIL receptor expression in human neutrophils. Immunology 111:186–194

Koga Y, Matsuzaki A, Suminoe A et al (2004) Neutrophil--derived TNF-related apoptosis-inducing ligand (TRAIL): a novel mechanism of antitumor effect by neutrophils. Cancer Res 64:1037–1043

Maianski NA, Maianski AN, Kuijpers TW et al (2004) Apoptosis of neutophils. Acta Haematol 111:56–66

Moudling DA, Quayle JA, Hart CA et al (1998) Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. Blood 92:2495–2502

Simon HU (2003) Neutrophil apoptosis pathways and their modifications in inflammation. Immunol Rev 193:101–110

Sobin LH, Wittekind C (1997) UICC TNM classification of malignant tumours, 5th edn. Springer, Berlin

Sprick MR, Walczak H (2004) The interplay between the Bcl-2 family and death receptor-mediated apoptosis. Biochim Biophys Acta 1644:125–132

Stoppacciaro A, Melani C, Parenza M et al (1993) Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T cell-produced interferon gamma. J Exp Med 178:151–161

Wang S, El-Deiry W (2003) TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 22:8628–8633