

TLRs and Bcl-2 family proteins in neutrophils of oral cavity cancer patients

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Abstract: Human neutrophils (PMNs), the cells engaged in the early phase of anti-tumor response, express TLR2 and TLR6 that can modulate the Bcl-2 family proteins, regulating the intrinsic apoptotic pathway in these cells. The expression of TLRs and Bcl-2 family is controlled by means of activating the transcriptional signaling pathways that involve the p38 MAP kinase. As previously described, PMNs from cancer patients exert accelerated apoptosis associated with decreased expression of anti-apoptotic Mcl-1 protein. In the present study we have been interested in establishing the involvement of TLR2 and TLR6, and p38 MAP kinase in the Mcl-1-modulated apoptosis in PMNs of oral cavity cancer patients. The expression of these proteins in neutrophils and autologous peripheral blood mononuclear cells (PBMCs) was analyzed by Western blot, the intensity of apoptosis was estimated by flow cytometry, caspase-9 activity by colorimetric assay, and the cytochrome c concentration by ELISA. The simultaneous decreased expression of examined TLRs receptors and Mcl-1 protein, associated with the acceleration of PMNs apoptosis, suggests that this process in PMNs controlled by Mcl-1 is dependent on the TLR2 and TLR6 signalling. Impaired TLRs expression can lead to insufficient activation of p38PAPK, resulting in low expression of antiapoptotic Mcl-1 protein responsible for shortened lifespan of the examined PMNs.

Key words: neutrophils, toll-like receptors, apoptosis, cancer

Introduction

Recent data suggest that Toll-like receptors (TLRs) participate in the development of cancer through direct influence on the growth of tumor cells, as well as through the modulation of antitumor activity and survival of the immune cells [1]. Neutrophils (PMNs), the primary effector cells of the early phase of the immune response to tumor, express the majority of the 11 human TLRs, which initiate different biological functions of these cells [2]. It has been reported that PMNs' stimulation through TLR2 causes an immediate defensive response, including *e.g.* cytokine production and secretion [2,3]. Detailed research has revealed that TLR2 is coexpressed with TLR6 on the neutrophils and has the ability to form heteromers with TLR6 to mediate the intracellular signaling, leading to survival or apoptotic cell death [4-7].

Two distinct apoptotic signaling pathways in human neutrophils have been identified: the intrinsic

pathway, regulated by members of the Bcl-2 family proteins and the extrinsic pathway, associated with TNF superfamily proteins involving FasL, TNF or TNF-related apoptosis-inducing ligand (TRAIL). Neutrophils constitutively express pro-apoptotic Bax and anti-apoptotic Mcl-1 proteins, belonging to Bcl-2 family [8]. It has been well established that Bax, by fusion with mitochondria upon apoptosis, results in the permeabilization of the outer membrane, with subsequent release of proapoptotic proteins, such as cytochrome c, Smac/DIABLO and HtrA2/Omi [8]. Cytochrome c binds to apoptotic protease-activating factor-1 (Apaf-1), which recruits procaspase-9. In this complex procaspase-9 undergoes an autoproteolytic transformation to caspase-9. Smac/Diablo and HtrA2/Omi are antagonists of inhibitors of apoptosis proteins (IAPs) that are required to relieve the inhibited caspases [9].

The expression of proapoptotic or antiapoptotic members of the Bcl-2 family in neutrophils is controlled by the activation of transcriptional signaling pathways that involve the mitogen - activated protein kinase p38 (p38MAPK) [10]. The same kinase is activated by the stimulation of TLRs receptors [7]. Additionally, TLRs can play a significant role in the regula-

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tion of Bcl-2 family proteins. It was reported that TLR2 and TLR6 activation can delay apoptosis through the modulation of anti-apoptotic Bcl-2 family proteins, associated with increased levels of Mcl-1 and A1 [7].

Our previous study showed the acceleration of apoptosis in PMNs derived from oral cavity cancer patients and suggested that this process may be dependent on the decreased expression of anti-apoptotic Mcl-1 protein, which accompanied the unchanged pro-apoptotic Bax expression [11]. In order to better understand the mechanisms responsible for shortened survival of neutrophils in this patient group we took interest in the involvement of TLR2 and TLR6, as well as p38 MAPK, in Mcl-1-mediated apoptosis in those cells. Additionally, to confirm the lack of engagement of pro-apoptotic proteins of Bcl-2 family in this process, we examined the expression of Smac/DIABLO proteins, cytochrome c release and caspase-9 activity, which are initiated not only by Bax but also by other proapoptotic proteins of Bcl-2 family.

Materials and methods

Sample material. The examination involved 20 patients (average age 49 years) with squamous cell carcinoma of oral cavity, undergoing treatment in the Department of Oral and Maxillofacial Surgery at Medical University of Bialystok. Examinations were carried out on patients before the treatment and 3 weeks after the surgical removal of the tumor mass. Patients did not receive any treatment or medication before the examination. No clinical signs of infection were observed in patients. The mean number of white cells in the blood of all patients before treatment was $6.18 \times 10^3/\mu\text{L}$ (from 3.8 to $8.95 \times 10^3/\mu\text{L}$), after treatment was $5.6 \times 10^3/\mu\text{L}$ (from 4.0 to $8.25 \times 10^3/\mu\text{L}$). The study included a healthy persons as control group ($n=15$) (mean 41.5 years). The study was approved by the Ethics Committee of the Medical University of Bialystok and all patients submitted their consent in writing.

Preparation of leukocytes. For the purpose of the examinations of the expression of TLR2, TLR6, Mcl-1, Bax, Smac/DIABLO and p38 MAPK in PMNs and in autologous PBMCs (for comparative purposes), the cells were isolated from anticoagulant venous blood. PMNs and PBMCs suspensions (5×10^6 cells/mL) with purity $>94\%$ were incubated for 20 h at 37°C .

Western blot analysis. The protein fractions of cells were analyzed for the presence of examined proteins by Western blot, as previously described [12].

Apoptosis evaluation. Annexin V binding of neutrophils was performed using an apoptosis detection kit (APOTESTTM - FITC, Nexins Research, Holland). Initially, 105 cells/ml of freshly isolated cells or incubated cells were labeled with FITC-conjugated Annexin V and propidium iodide (PI) for 20 min at $+4^\circ\text{C}$. The samples were analyzed by two-color flow cytometry using CellQuest Analysis software (Becton Dickinson, Epics Coulter, USA). Results were reported in the form of the percentage of Annexin V-positive cells, which reflects the relative proportion of apoptotic cells. The percentage of cells positive for PI and Annexin V-FITC and PI were considered as necrotic.

Caspase-9 activity. Caspase-9 activity was measured using Caspase-8 Colorimetric Assay (R&D Systems). Cell lysates were test-

ed for protease activity by means of 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, quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction.

Cytochrome c concentration. Cytochrome c concentrations in cell lysates were assessed using ELISA kit (R&D systems, Minneapolis, USA).

Results and discussion

The results of the present study appear to confirm the role of TLR2 and TLR6 receptors in the apoptosis process in PMNs from oral cavity cancer patients. Decreased expressions of both TLR2 and TLR6, analyzed by Western blot and quantified using LabImage 1 D gel software (Fig. 1), were accompanying the increased intensity of apoptosis in the PMNs from patients before treatment, in comparison to the control (12.6% and 0.65% respectively). TLRs expression in PMNs and autologous PBMCs was on the same level. However, the number of PBMCs with apoptotic features in patients before and after the treatment was similar to that of the control group (0.91% and 1.2% respectively). The simultaneous decreased expression of both TLRs and the decreased expression of Mcl-1 protein observed earlier appear to confirm the suggestion that apoptosis of PMNs from cancer patients controlled by Mcl-1 is dependent on the TLR2 and TLR6 signalling [11]. In contrast to PMNs, the absence of changes in Mcl-1 expression in autologous PBMCs might be associated with the unchanged intensity of apoptosis in those cells in patients before and after treatment (1.6%).

Impaired TLRs expression can lead to an insufficient activation of p38 MAPK, involved in the regulation of cellular Mcl-1 expression [1,10,13]. In the examined PMNs we found a lowered expression of phospho-p38MAPK, which is a sign of p38MAPK activation (Fig. 2). The observation above suggests that the low expression of TLRs and p38MAPK activity leads to the low expression of antiapoptotic Mcl-1 protein, which is responsible for the shortened lifespan of the examined PMNs. The absence of Mcl-1 activity in PMNs of cancer patients may be associated with the unchanged concentration of apoptogenic cytochrome c observed in those cells ($7.07 \text{ ng/ml} \pm 4.3 \text{ ng/ml}$ and $8.84 \text{ ng/ml} \pm 3.7 \text{ ng/ml}$, respectively). It is known that Mcl-1 interacts with tBid and impairs its ability to induce cytochrome c release from mitochondria. Since cytochrome c participates in the formation of caspase-9, its unchanged concentration in PMNs from cancer patients, may be associated with the absence of changes in caspase-9 activity, demonstrated in these cells (0.182 ± 0.089 and 0.200 ± 0.073 , respectively). The activity of this enzyme in autologous PBMCs

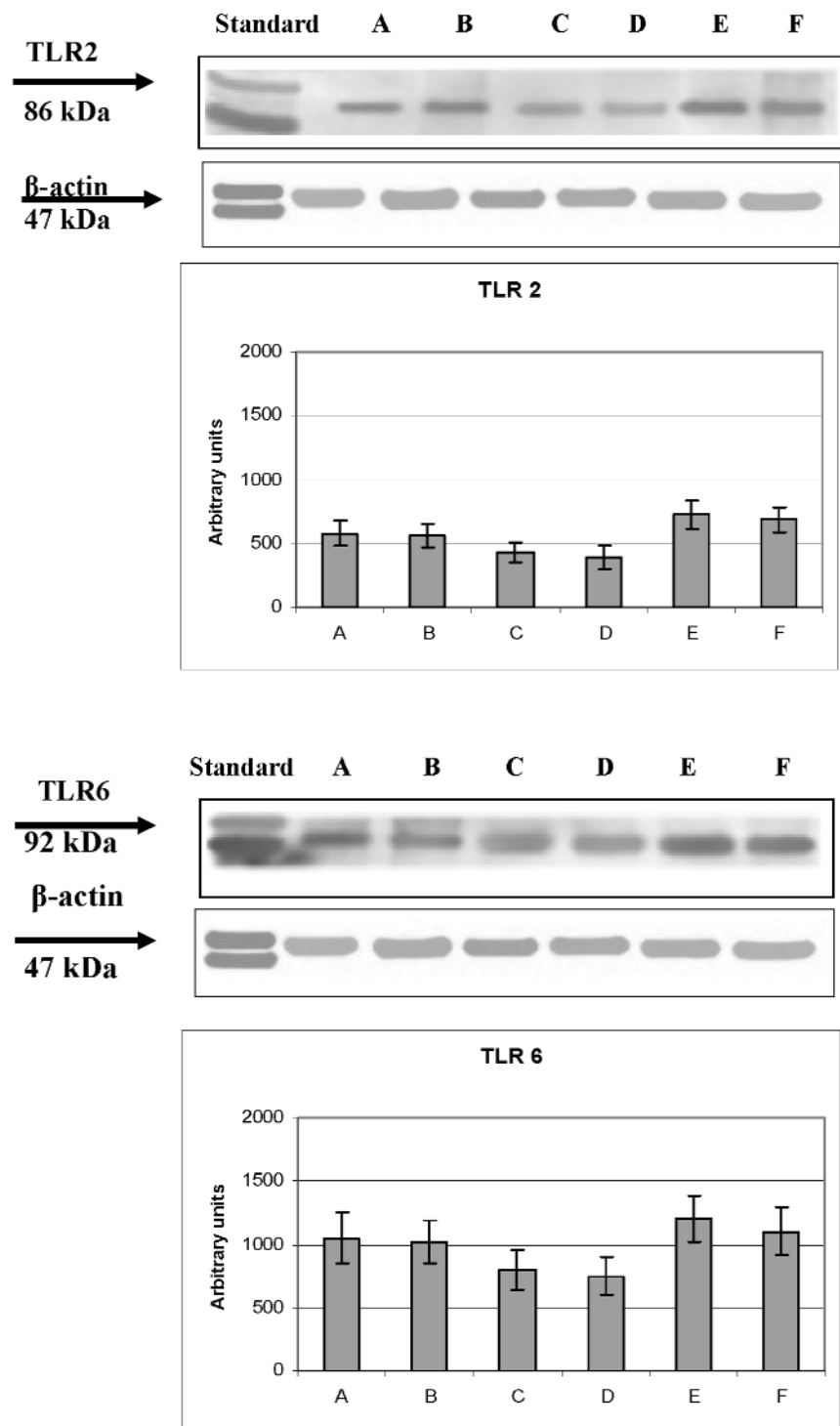


Fig. 1. Western blot analysis of TLR2 and TLR6 proteins expression in PMN and PBMC of control and oral cavity cancer patients: A - PMN of control, B - PBMC of control, C - PMN of patients before treatment, D - PBMC of patients before treatment, E - PMN of patients after treatment, F - PBMC of patients after treatment. The diagram below is shown statistical analysis of Western blot for five independent samples of blood. Bands intensity was quantified using LabImage 1 D gel software and presented in arbitrary units. *Statistical differences between PMN and PBMC ($p < 0.05$). *Statistical differences between patients and control ($p < 0.05$). ^bStatistical differences between patients before and after treatment ($p < 0.05$)

lysates of patients (0.152 ± 0.076 , 0.147 ± 0.09) was similar to the control group level (0.142 ± 0.064).

The decreased expression of TLR2 and TLR6 in PMNs, despite its effect on the apoptosis in these cells, may have other implications for the immune defense of cancer patients. The impaired expression of both TLRs can lead to disturbances in the secretion of different anti-tumor molecules by neutrophils, such as

TNF- α or TRAIL [7,14]. TRAIL induces apoptosis in a number of tumor cells through a complex system of two death receptors TRAIL-R1(DR4) and TRAIL-R2(DR5) that are expressed on head and neck squamous carcinoma cells (HNSCC), making them sensitive to TRAIL-mediated apoptosis [15]. The decreased secretion of TRAIL by PMNs and PBMCs from advanced oral cavity cancer, demonstrated in earlier

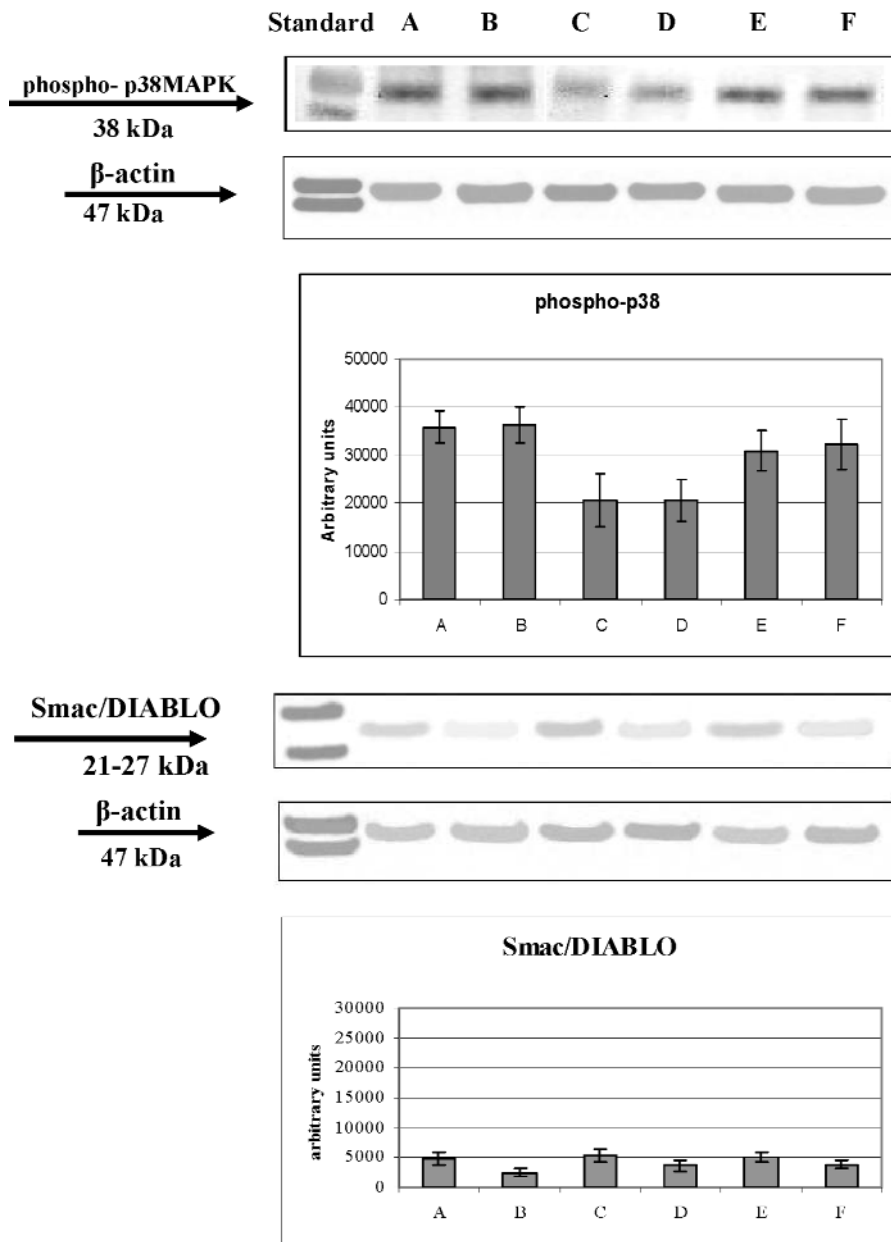


Fig. 2. Western blot analysis of phospho-p38 and Smac/Diablo proteins expression in PMN and PBMC of control and oral cavity cancer patients: A – PMN of control, B – PBMC of control, C – PMN of patients before treatment, D – PBMC of patients before treatment, E – PMN of patients after treatment, F – PBMC of patients after treatment. The diagram below is shown statistical analysis of Western blot of Bax and Smac/DIABLO from five independent samples of blood. Bands intensity was quantified using LabImage 1 D gel software and presented in arbitrary units. *Statistical differences between PMN and PBMC ($p < 0.05$). *Statistical differences between patients and control ($p < 0.05$). ^bStatistical differences between patients before and after treatment ($p < 0.05$).

study, may be responsible for impaired apoptosis of the tumor cells [12]. It remains unclear if the changes in the release of this proapoptotic molecule are directly associated with TLR2 and TLR6 expression in the examined leukocytes.

In patients after the surgical treatment the increased expression of TLR2 and TLR6, associated with the increased expression of Mcl-1 protein and simultaneous delay of apoptosis in PMNs, suggests a transient deficit of these proteins in patients before treatment, caused by a direct or indirect activity of the tumor cells. It was shown that tumor cells, including HNSCC cells, can release immunosuppression cytokines, such

as VEGF, which inhibit immune cells activation and impair tumor-specific immunity [16].

The comparison of the results obtained in patients before and after treatment appears to confirm the lack of engagement of proapoptotic proteins belonging to Bcl-2 family in the apoptotic process in PMNs. Despite the lack of alterations in cytochrome c concentrations and caspase-9 activity, the expression of Smac/DIABLO in PMNs and PBMCs lysates of patients and the control were on the same level (Fig. 2).

To conclude based on the relations between the estimated TLRs receptors, p38MAPK and Mcl-1 protein expression in confrontation with the intensity of

apoptosis in PMNs of patients, we can confirm the influence of these molecules on the shortened lifespan of neutrophils in oral cavity cancer patients. Furthermore, PMNs from oral cavity cancer patients appears to be more sensitive in tumor microenvironment than autologous PBMCs. Further research is necessary to explain the dependence between the observed changes and the clinical status of patients that may have a potential prognostic significance and also to contribute to the development a new immunotherapy strategy for oral cavity cancer based on the modulation of TLRs.

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