Abstract. Background/Aim: Endothelial microparticles (EMP) are small vesicles which are released from the endothelium and contribute to blood coagulation activation in various clinical settings. The aim of this study was to examine whether EMP influence blood coagulation activation in cancer patients during radiotherapy/radiochemotherapy (RT/RCT). Materials and Methods: Sixteen head and neck cancer (HNC) patients undergoing RT/RCT and 10 controls were examined. EMP and thrombin-antithrombin complex (TAT) were measured by flow cytometry and enzyme-linked immunosorbent assay (ELISA), respectively. Tissue factor-positive EMP (TF+EMP) were defined as CD31+/CD142–/CD42b–. Results: TF+EMP were significantly elevated in HNC patients before RT/RCT (T0) (1299±1154/μl), one day after RT/RCT (T1d) (1257±603/μl) and 3 months after RT/RCT (T3m) (1289±372/μl) compared to controls (688±647/μl). TF+EMP levels at T0/T1d and T0, as well as at T1d and T3m were not significantly different. TAT levels at T0 and T1d did not differ significantly but at T3m were significantly lower compared to T0 and T1d. TF+EMP and TAT concentrations were not significantly correlated at T0 (r=0.058; p=0.828), T1d (r=0.373, p=0.154) and T3m (r=-0.302, p=0.204). Conclusion: TF+EMP may not contribute to hemostatic abnormalities in HNC patients.

Endothelial microparticles (EMP) are small vesicles released from the endothelium upon activation or apoptosis (1). Endothelium-derived microparticles represent about 10-15% of the total microparticles (MP) in blood of healthy individuals. Their concentration ranges from 1 to 70 x 10³/ml and their diameter varies from 0.1-2 μm (2, 3). Endothelial microparticles are characterized by the expression of adhesion molecules specific for mature endothelial cells (ECs), such as CD31 (platelet-endothelial cell adhesion molecule, PECAM), CD62E (E-selectin), CD54 (intracellular adhesion molecule-1, ICAM-1) and CD62P (P-selectin). They also express CD105 (endoglin, a proliferation-associated protein), CD144 (vascular endothelial-cadherin), CD146 (Sendo1, an endothelial junctional protein), von Willebrand Factor (vWF), and phosphatidylserine (PS) (2, 3). A number of in vitro studies have demonstrated that EMP are formed after EC stimulation by inflammatory cytokines (including tumor necrosis factor – TNF-α), bacterial lipopolysaccharides, reactive oxygen species, plasminogen activator inhibitor, thrombin, camptothecin and C-reactive protein (CRP) (4, 5). Endothelial microparticles released from activated or apoptotic EC are emerging as useful markers for detection of ECs dysfunction (6, 7).

The EMP level in circulating blood depends on sex (higher levels are observed in women), menstrual cycle phase and presence of some pathologic conditions (1). In vivo, EMP were found in the peripheral blood of patients with cardiovascular disorders, such as acute coronary syndrome, atherosclerosis and congestive heart failure. In addition, EMP levels were altered in diseases well known to be accompanied by ECs dysfunction, such as metabolic syndrome, obesity, diabetes mellitus, stroke, sepsis, and advanced stages of renal failure (6, 8, 9). Moreover, higher level of EMP was reported in hematological disorders e.g. lupus anticoagulant, sickle cell disease, antiphospholipid syndrome and venous thromboembolism (10, 11).

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Key Words: Endothelial microparticles, thrombin-antithrombin complex, tissue factor, head and neck cancer, coagulation activation.

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The current standard of treatment for newly diagnosed head and neck cancer (HNC) patients is surgery followed by radiotherapy (RT) or radiochemotherapy (RCT). RT alone or concomitant RCT. However, during RT not only cancer cells but also normal tissues (among them - ECs localized in the irradiated volume) are exposed to high-energy ionizing radiation and, as a result, patients experience symptoms associated with tissue damage for few weeks, months or years after RT. Early (acute) postradiation reaction (e.g. dermatitis, mucositis) is associated with inflammation and cytokine-mediated responses (12). The symptoms produced by radiotherapy occur 2-3 weeks into the treatment with the greatest intensity at the end of treatment and after its completion. They usually resolve 6-8 weeks after the treatment and they disappear up to 3 months in most cases (12). Oropharyngeal and laryngeal cancer patients treated with RT were found to be at higher risk of developing venous thromboembolism (VTE) compared to their radiation-spared counterparts (13, 14). The prevalence of VTE among HNC patients undergoing surgery is 1.4-5.8%, and can be as high as 13% when asymptomatic VTE cases are included (15). There is growing evidence that TF enriched-EMP could be a potential marker of procoagulant state in diseases associated with damage of blood vessel endothelium (3, 16, 17). Nevertheless, there is no data concerning the RT/RCT influence on EMP formation in vivo as well as the potential impact of EMPs on blood coagulation activation in HNC patients during RT/RCT.

The aim of the study was to determine the potential EMP contribution to hemostatic abnormalities in HNC patients undergoing RT/RCT.

Materials and Methods

Blood samples were obtained from sixteen HNC patients diagnosed with squamous cell carcinoma in IIB-IVA clinical stages before RT (no acute radiation), one day after its completion (clinically exacerbated radiation-induced inflammation) and 3 months after the treatment (when the inflammation is resolved). Patients with conditions known or suspected to increase EMP level such as lupus anticoagulant, sickle cell disease, antiphospholipid syndrome, venous thromboembolism, metabolic syndrome, obesity, diabetes mellitus, stroke, sepsis, and advanced stages of renal failure were excluded. All patients suffered from mild hypertension. Patients were treated with RT alone, adjuvant RT or RT combined with chemotherapy (cisplatin 100 mg/m² every 21 days or 40 mg/m² every 1 week). Intensity modulated radiotherapy (IMRT) with high energy 6MV photons to a total dose of 60-67.5 Gy in 30 fractions (2-2.25 Gy per day) was administered. Patients’ characteristics are shown in Table I. Written informed consent was obtained from the patients and the study protocol was approved by the Bioethics Committee of Medical University in Bialystok, Poland, according to the Guidelines for Good Clinical Practice (approval number - R-I-002/376/2010). Control group consisted of 10 healthy individuals (7 females and 3 males).

Five ml of venous blood were collected in Monovette – Sarstedt tubes containing 3.8% sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA) using 21-gauge needles (Becton Dickinson) from all patients. Two steps of centrifugation were used in order to isolate EMP from whole blood. Firstly, blood samples were centrifuged for 15 min at 500 g (removal of erythrocytes and leukocytes) to prepare platelet-rich plasma (PRP). The PRP was further centrifuged for 5 min at 14,000 g to obtain platelet-poor plasma (PPP). Both centrifugations were performed at room temperature (+24°C), to avoid activation of platelets and plug generation at lower temperature. Plasma samples were not frozen because of harmful influence of low temperature on EMP vitality. The storage time of samples never exceeded 1 h after venipuncture. Endothelial microparticles were defined as particles bearing antigens such as CD31, CD62E, measuring below 1.5 μm, and were quantified with flow cytometry using two panels of monoclonal antibodies (CD31/CD62E/CD42b and CD31/CD62E/CD142). Monoclonal antibody directed to CD42b was used to exclude EMP released from platelets and megakaryocytes, whereas anti-CD142 was required for detection of tissue factor (TF)-bearing EMP.

The following monoclonal antibodies purchased from Becton Dickinson, were used in the study: CD31 stained with FITC, CD62E stained with APC, PE-Cy5-labeled anti-human CD42b, PE-labeled anti-human CD142 and kappa isotype control: FITC-labeled mouse IgG1, APC-labeled mouse IgG, PE-Cy5-labeled mouse IgG1, PE-labeled mouse IgG1. Moreover, Cell WASH, fluorescence-activated cell sorting (FACS) lysing solution, PBS wash buffer, FACS Flow Sheath Fluid, and FACS lysing solution were utilized in the study.

| Clinical factors          | n=16 | % |
|---------------------------|------|---|
| Gender                    |      |   |
| Female                    | 5    | 31|
| Male                      | 11   | 69|
| Age (years)               |      |   |
| 31-50                     | 2    | 12|
| 51-70                     | 13   | 82|
| >70                       | 1    | 6 |
| Localization              |      |   |
| Lip, tongue               | 2    | 12.5|
| Floor of oral cavity and palate | 4   | 25|
| Hyopharynx                | 3    | 19|
| Tonsil                    | 2    | 12.5|
| Indeterminate part of oral cavity | 5  | 31|
| CS                        |      |   |
| II B                      | 1    | 6 |
| III                       | 4    | 25|
| IV A                      | 11   | 69|
| Grade                     |      |   |
| G1                        | 2    | 12.5|
| G2                        | 12   | 75|
| G3                        | 2    | 12.5|
| Treatment                 |      |   |
| RT alone                  | 7    | 44|
| RCT                       | 9    | 56|

G: Grade; CS: clinical stage according to TNM classification; RT: radiotherapy; RCT: radiochemotherapy.
Aliquots of PPP (30 μl) were incubated with 10 μl of monoclonal antibodies in the dark. After 20 min of incubation at room temperature, the samples were gently shaken (orbital shaker, 120 rpm) and phosphate-buffered saline (PBS) was added. Samples of 18 μl each were aspirated and counted in a 30-sec run at medium setting. The detection of particles was triggered by a fluorescent signal greater than a predetermined value. Light scatter and fluorescence channels were set at logarithmic gain. The fluorescence-positive particles were further separated on another histogram based on size (forward light scatter). Flow cytometer counts were converted to a number of EMP per μl. Endothelial microparticles were analyzed by flow cytometry FACS Calibur (Becton Dickinson) in Regional Centre for Transfusion Medicine in Bialystok, Poland, according to a protocol used in a previous study (18).

To assess thrombin-antithrombin (TAT) plasma complex concentration (indicating activation of blood coagulation), IMUBIND TAT (American Diagnostica, Stamford, CT, USA) was used. Five-milliliter samples of venous blood from HNC patients undergoing RT/RCT were obtained (before treatment, one day after its completion and 3 months after the treatment) and collected in Monovette – Sarstedt tubes containing 3.8% sodium citrate (Becton Dickinson). Blood samples were centrifuged for 15 min at 1500 × g to receive plasma, which in the next step was frozen (≤ –20˚C).

Statistical analysis. Given the non-normal distribution (Shapiro–Wilk test) of the EMP concentrations, values were expressed as mean, median and standard deviation. The Wilcoxon rank sum test for dependent data was used to evaluate significance between EMP levels in different points of the study. Correlation between TF-positive EMP and TAT was measured using Spearman test. Statistical significance was defined as p-value <0.05. Analyses were performed with the statistical package STATISTICA 10 and Microsoft Excel 2010.

Results

TF-bearing EMP in HNC patients. Tissue factor positive EMP concentration was significantly higher in plasma of HNC patients before RT/RCT (1299±1154 EMP/μl), one day after RT completion (1257±603 EMP/μl) and 3 months after the treatment (1289±372 EMP/μl) compared to healthy participants (688±647 EMP/μl) (p<0.05) (Figure 1). Interestingly, there was no significant difference between the levels of TF-bearing EMP after RT/RCT completion and pre-treatment levels. Moreover, there was no significant difference in TF-bearing EMP levels one day after RT/RCT completion compared to 3 months after the treatment (Table II).

TAT concentration in HNC patients. Plasma TAT concentration was significantly higher in HNC patients before RT/RCT (1.41±1.79 ng/ml) in comparison to control group (0.77±0.57 ng/ml). There was no significant difference in TAT level before and one day after RT/RCT (1.7±0.89 ng/ml). Plasma TAT level 3 months after RT/RCT completion was significantly lower (0.68±1.31 ng/ml) in comparison to TAT concentration before treatment and one day after its completion – p<0.05 (Figure 2).

| Time of assessment | EMP (CD31+/CD142+/CD42b) (quantity/μl) |
|--------------------|----------------------------------------|
| Control group      | 688±647                                |
| Before RT/RCT      | 1299±1154                              |
| One day after RT/RCT completion | 1257±603                              |
| 3 months after RT/RCT | 1289±372                              |

CD31: EMP surface antigens; CD142: tissue factor; CD42b: platelet and megakaryocyte surface antigens; X: mean value; SD: standard deviation; Me: median. *Statistically significant difference in comparison to control group.
TF-positive EMP and TAT. There was no significant correlation between the level of TF-positive EMP (CD31+/CD142+/CD42−) and TAT concentration in the blood of HNC patients before RT/RCT (r=0.058, p=0.828), one day after RT/RCT (r=0.373, p=0.154) and 3 months after the treatment completion (r=−0.302, p=0.204) (Figure 3).

Discussion

The activation of blood coagulation in HNC patients undergoing RT/RCT is most likely multifactorial and involves inflammation, platelet recruitment, delayed re-endothelialization and overexpression of TF (19). Aside from VTE, blood coagulation can contribute to a variety of other pathologic processes, such as metastasis, tumor growth and tumor angiogenesis (20, 21).

Endothelium-derived microparticles are proven to play an important role in the pathogenesis of thrombotic disorders via multiple ways, e.g. TF expression (main procoagulant in cancer) (10, 22) and providing negatively charged phospholipid surface (3, 22). Phospholipids present on EMP surface facilitate binding of coagulation factors and promote the formation and activity of coagulation enzyme complexes (3, 22, 23). Injury or stimulation of ECs contribute to TF exposure to coagulation factor VII, with subsequent TF/VIIa complex formation and initiation of coagulation cascade leading to thrombin generation and fibrin formation (10). Endothelial microparticles enriched with TF are also involved in procoagulant response (10, 24). Furthermore, TF-positive EMP exhibit endothelial adhesive molecules, which allows for binding to other cell types, such as monocytes and platelets, and likely enables TF transferring onto their surface (25, 26).

In addition, EMP carry von Willebrand Factor (vWF), an adhesive protein which interacts with platelets and contributes to initiation and progression of thrombus formation (27).

It has been proven that TF-positive vesicles released from endothelium contribute to hypercoagulable state in cancer (28). Ionizing radiation also contributes to blood coagulation activation (29). A study of Szotowski and colleagues (30) showed that ionizing radiation of 5-10 Gy increased the

Figure 3. Correlation between tissue factor-positive endothelial microparticles (EMP) levels and concentration of thrombin-antithrombin complex (TAT) in head and neck cancer patients undergoing radiotherapy/radiochemotherapy (RT/RCT).
release of EMP-associated TF from human umbilical vein endothelial cells (HUVEC) in vitro. However, in our study the level of TF-bearing EMP in HNC patients remained unchanged after RT/RCT. The reason for these conflicting results remains unclear, however, it is possible that higher TF-positive EMP concentrations are found mainly in irradiated tissues and not necessarily in peripheral blood. The dose of radiation can also play a role, as our patients were treated with 2-2.5 Gy per day which is 2.5-5 times less than a single radiation dose in the above-mentioned in vitro study. Unfortunately, there is no clinical data on RT influence on TF-positive EMP release, therefore our results could not be compared to other studies performed in clinical setting.

Compared to healthy individuals, the levels of TF-bearing EMP were higher in HNC patients both before and after RT/RCT, which is consistent with the findings of a study by Campello et al. (31) showing higher levels of EMP and TF-positive MP in cancer patients than in healthy controls. Although TF-bearing MP levels were reported to be higher in cancer patients with a diagnosis of VTE than without it (31), it remains unclear whether elevated TF-positive MP levels are a cause or a consequence of VTE. Given that thrombin inhibitors were found to prevent the increases in circulating tumor-induced TF-positive MPs (32), thrombin generated during VTE event may be one of the factors contributing to TF-positive MPs release.

Interestingly, the present study revealed no correlation between TF-positive EMP and TAT levels before and after RT/RCT suggesting that EMP may not contribute to blood coagulation activation. Of note, EMP also express anticoagulant factors such as thrombomodulin and protein C receptors (33), therefore, the overall coagulable properties of EMP in cancer patients may depend on the balance between pro- and anticoagulation factors found within EMP (34).

Importantly, EMP subpopulations expressing various antigens require distinct methods of their isolation from the whole blood (centrifugation protocol) which makes it difficult to compare the results of various studies. Namely, EMP formed as a result of ECs activation via TNF-α stimulation are characterized by expression of inducible endothelial markers, such as CD62E, whereas EMP released during apoptosis are more likely to constitutively express endothelial cell markers - CD31 (2, 5).

In conclusion, our study does not provide evidence for RT/RCT influence on rapid procoagulant EMP release or EMP contribution to the activation of blood coagulation in HNC patients undergoing RT/RCT. Further research and study group enlargement is needed to define whether concentrations of EMP in HNC patients are sufficient to initiate or augment blood coagulation.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors’ Contributions

Ewa Sierko – Concept of the study, supervision, interpreting results, writing the manuscript, approval of the text of the manuscript.

Monika Sobierska – collecting material, performing study, interpreting the results, writing the manuscript, performing experiments, approval of the manuscript.

Ewa Zabrocka – interpreting the results, writing the manuscript, performing experiments, approval of the manuscript.

Marta Myśliwiec – performing experiments, literature searching, approval of the manuscript.

Joanna Krużewska – performing experiments, literature searching, approval of the manuscript.

Alina Lipska – performing experiments, approval of the manuscript.

Piotr Radziwon – supervision on the experiments, approval of the manuscript.

Marek Z. Wojtkiewicz – concept of the study, supervision, approval of text of manuscript.

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