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Distribution of circulating natural killer cells and T lymphocytes in head and neck squamous cell carcinoma

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ABSTRACT

Objective: Natural killer (NK) cells are capable of eliminating malignantly transformed cells without prior sensitization. In contrast to NK-cells, T lymphocytes possess antitumourous activity that is restricted to major histocompatibility complex (MHC) recognition. The aim of this study was to determine the causes of the different distributions of these cell types in the peripheral blood of patients with head and neck squamous cell carcinomas (HNSCC).
Methods: A cohort of 105 subjects was divided into three clinical groups: non-treated HNSCC patients, treated relapse-free HNSCC patients and healthy control subjects. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood, subsets were depleted, flow cytometric counts were made and subsequently correlation analyses with clinical parameters were performed.
Results: Treated relapse-free HNSCC patients have a significantly increased mean proportion of NK-cells in PBMC of 26.39% ($p < 0.001$), whereas T lymphocytes and natural killer-T-(NKT) cells of treated patients have a significantly decreased mean proportion in PBMC of 55.15% ($p < 0.05$) at least 12 months after treatment. This inverse redistribution of these two subsets is reflected in a significantly increased mean NK/T-ratio of 0.54 ($p < 0.05$) in treated patients. The NK/T-ratio correlates with the systemic invasiveness of the type of therapy patients undergo and is highest after surgery with adjuvant radiochemotherapy (0.64, $r_s = 0.334$, $p < 0.01$). This appears to be a post-therapeutic long-term effect in treated patients, as they had a mean relapse-free period until venous puncture of 47.9 months in our study. We also demonstrated age-dependent changes in the peripheral distribution of T- and NK-cells.
Conclusion: These findings reveal new aspects in understanding tumour biology and interactions with the cellular immune system which provide novel starting points for further research.

1. Introduction

Cancer cells can be detected and eliminated by the immune system, described as 'immunological surveillance' by Burnet [1], and this is mainly restricted to the ability of natural killer (NK) cells and T-lymphocytes. Being part of the innate cellular immune system, NK-cells (following cluster of differentiation (CD) nomenclature, CD3−, CD16+, CD56+) have an approximate proportion of 6–29% in peripheral blood mononuclear cells (PBMC) in healthy individuals. T-lymphocytes (CD3+), including subsets such as natural killer-T (NKT) cells (together subsequently named T-cells), are included among the adaptive immune system and have a proportion in PBMC of approximately 61–85% in healthy subjects [2]. In contrast to T-cells, NK-cells are not subject to major histocompatibility complex (MHC) restriction and they are capable of mediating cytolytic reactions without prior sensitization in virus-infected or malignantly transformed cells in different ways [3]. NK-cells recognize target cells by reduced or absence of autologous MHC-I-expression ('missing-self' hypothesis by Klas Kärre [4]) disabling NK-receptors (killer-cell immunoglobulin-like receptors and CD94/NKG2A receptors) to initiate cytolysis inhibiting signal cascades. The natural cytotoxicity of NK-cells is mediated via the perforin/granzyme mechanism. Other apoptosis-inducing mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), FAS-L or TRAIL-R ligand activation have been discovered in recent times [5].

On the basis of surface molecule expression, T-lymphocytes can be divided into cytotoxic CD8+ and CD4+ helper cells, different

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regulatory T-cells and NKT cells. CD8+ lymphocytes lyse tumour cells with aberrant peptides MHC-I-dependently. CD4+ helper cells regulate antitumourous activity by cytokine production passed to MHC-II-bound antigen recognition. In particular, those cytokines produced by T helper-1 cells such as interleukin (IL)-12 and interferon-γ (IFN-γ) have a stimulating effect on cellular immune response [6].

NKT-cells are characterized by both the presence of a T-cell receptor as well as CD56 and/or CD161 and they can be CD4+ and/or CD8+. They contribute decisively to immune regulation by cytokine production (e.g. IFN-γ, IL-4, tumour necrosis factor) and have an approximate proportion of 0.2% in peripheral CD3+ cells, and 0.1% in PBMC of healthy individuals [7,8].

The interactions of tumour cells and the immune system are highly complex and they are subject to a variety of control circuits of partly cytokine-mediated, cellular (T-cells, NK-cells, dendritic cells, eosinophils, macrophages) and humoral (mainly plasma cells) mechanisms [6,9]. The extent of the mutual influence of HNSCC on the immune system has been controversially debated. In addition to anticancer activity, knowledge of defective immune surveillance and tumour cell escape mechanisms is fundamental in understanding HNSCC tumour biology [10].

There have been several published studies on the distribution of lymphocyte subpopulations in healthy individuals [2,11–13]. Likewise, earlier work showed cell changes with different effects on the prognosis of cancer patients. For example, a decreased percentage of CD8+ lymphocytes in the peripheral blood of cancer patients seems to be associated with a poorer prognosis [14]. Lowered levels of NKT cells in peripheral blood also appear to correlate with poorer prognosis of cancer patients [15]. Simultaneously, a decreased percentage of NK cells is considered as a possible consequence of immune escape mechanisms in HNSCC [16].

In this study, we present flow cytometric analyses of PBMC of HNSCC patients with and without treatment in direct comparison to healthy subjects and demonstrate the possible causes of the different distributions of subsets in peripheral blood by correlation analyses with clinical parameters.

2. Materials and methods

2.1. Patients

The study was conducted in accordance with the revised version of the Helsinki Declaration. The study design was approved by the Ethics Committee of the General Medical Council of Mecklenburg Western Pomerania. All patients gave written consent.

We investigated a total of 105 subjects who were divided into three clinical groups:

- 'healthy': healthy control subjects
- 'non-treated': patients with HNSCC, no therapy up to that point
- 'treated': patients with status after HNSCC, no evidence of locoregional recurrence (relapse-free ≥ 12 months).

All study participants were at least 18 years old and had no malignant, haematological or underlying autoimmune disease. In the cohort of 87 men (82.9%) and 18 women (17.1%), the mean age of the subjects was 57.9 ± 10.2 years. The group 'healthy' consisted of 15 male and 7 female subjects ($n = 22$) with a mean age of 51.1 ± 12.3 years. The 'non-treated' patients ($n = 20$, males 18, females 2) had a mean age of 55.9 ± 9.3 years, whereas the group 'treated' consisted of 54 male and 9 female subjects ($n = 63$) with a mean age of 60.9 ± 8.3 years.

All tumour patients in the groups 'non-treated' and 'treated' ($n = 83$) had a histologically confirmed HNSCC with certain degrees of differentiation (G1, $n = 2$, 2.4%; G2, $n = 62$, 74.7%; G3, $n = 16$, 19.3%; G4, $n = 3$, 3.6%). The tumour staging including regional and distant metastases was carried out according to the 7th edition of the TNM classification by the International Union Against Cancer (UICC) 2010 [17]. More than two-thirds of all cancer patients ($n = 59$, 71.1%) had an advanced tumour stage (III–IV).

The mean relapse-free period after therapy until venous puncture in the group 'treated' was 47.9 months (range from 12 to 276 months).

The probands' basic clinical parameters are described in Tables 1 and 2.

2.2. Cell depletion and counting

Peripheral venous blood (37.5 ml) was obtained under sterile conditions on Wednesdays between 9 a.m. and 12 a.m., local time. The samples were collected in EDTA blood collection tubes (Serum-Monovette 7.5 ml K3E, 1.6 mg EDTA/ml; Blut, Sarstedt, Nümbrecht, Germany). Then isolation of PBMC by density gradient centrifugation was carried out over a Ficoll-Paque Plus (Amersham Bioscience, Buckinghamshire, UK) gradient according to the manufacturer's instructions. After addition of PBS (phosphate-buffered saline tablets, Sigma–Aldrich, Seelze, Germany) and centrifugation, the PBMC were isolated. Following antibody conjugation (CD3 MicroBeads and CD56 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany), lymphocytes were separated by immunomagnetic cell depletion using MACS (magnetic activated cell sorting) technology into CD3+ (T-cells) and CD3−/CD56+ (NK-cells) that were used for further immunological investigations.

PBMC were then labelled with fluorochrome-conjugated antibodies (Simultest CD3/CD16 + CD56 Reagenz; Becton Dickinson, San Jose, CA, USA). Afterwards, the cells were counted and characterized in terms of size, granularity and emission maxima of the selectively conjugated dyes phycoerythrin (CD16+, CD56+) and fluorescein isothiocyanate (CD3+) by flow cytometry (FACSCalibur Flow Cytometer; Becton Dickinson, San Jose, CA, USA). FACS (fluorescent activated cell sorting) immunophenotyping enables counting and differentiation of NK-cells and T-cells. We counted 10,000 events each. The tabular and graphical display of the results was performed with CellQuest Pro Software (Vers. 4.0.2; Becton Dickinson, San Jose, CA, USA) on a Power MAC G4 (Apple, Cupertino, CA, USA).

For closer antibody specifications for cell depletion please refer to the product details or contact the author of this paper.

2.3. Statistical analyses

Tests for normal distribution of results were performed using the Kolmogorov–Smirnov test. Univariate analyses of variance (ANOVA) for group comparisons were carried out whenever appropriate using the Bonferroni test. For correlation analyses

Table 1
Distribution of tumour locations.

n		Clinical group		Total
		Non-treated	Treated	
Location	Larynx	8	25	33
	Oropharynx	8	22	30
	Hypopharynx	2	9	11
	Multi level[a]	2	7	9
Total		20	63	83

[a] Oropharynx/larynx: $n = 4$; oro-/hypopharynx: $n = 3$; oropharynx/larynx/hypopharynx: $n = 2$.