Tissue detection of natural killer cells in laryngeal carcinoma

Original article

A. C. LAZARIS[1*], J. V. SEGAS[2], T. P. NIKOLOPOULOS[2], E. S. PATSOURIS[1]

First Department of Pathology[1] and Department of Otolaryngology[2], School of Medicine, National and Kapodistrian University of Athens, 75, Mikras Asias St., (Goudi) GR – 115 27 Athens, Greece, e-mail: alazaris@med.uoa.gr

Received November 12, 2006

Natural Killer (NK) cells have gained much attention as potential cells in antitumor immune defense mechanisms. In a group of 31 patients with surgically treated squamous cell laryngeal carcinoma, NK cell presence was semiquantitatively assessed by means of immunohistochemistry. A panel of three monoclonal antibodies including anti-CD16, was applied on frozen tissue sections. High CD 16$^+$ cell presence was more frequently detected in poorly differentiated carcinomas (in 6 out of 14 cases) by comparison to carcinomas of high to moderate degrees of differentiation (in 1 out of 16 cases, p=0.031). No other clinicopathological variable appeared to influence NK cell presence in the examined specimens. No relation between NK cell detection and relapse-free survival emerged. Poorly differentiated laryngeal cancer cells appear to trigger off a greater NK cell tissue response than well and moderately differentiated cancer cells; however, the potential prognostic impact of this observation remains to be established.

Key words: Natural killer cells; laryngeal cancer; immunohistochemistry

Among immune cells surrounding tumor tissues, Natural Killer [NK] cells may provide the first line of defence against many tumors. NK cells are morphologically similar to lymphocytes and have cytotoxic capacity due to granules that contain perforins and granzymes. NK cells are so called because they can kill certain targets without sensitization and without Major Histocompatibility Complex [MHC] restriction [1]. Because a wide variety of unrelated tumors can be lysed by NK cells without apparent specificity, it appears that the target antigens recognized by NK cells might be highly conserved tissue antigens whose expression is enhanced or altered on malignantly transformed cells.

Squamous cell carcinomas of the head and neck are characterized by a dense infiltrate of mononuclear cells, which probably act in a very complex immunological system. In laryngeal carcinoma, the presence of a high inflammatory reaction surrounding the tumor has so far been correlated with decreased incidence of nodal metastasis [2]. Cells bearing the NK cell phenotype along with CD8$^+$ cells have been reported to be the most frequently encountered immune cells in laryngeal cancer mainly within the tumor mass but at low or nil state of activation [3]. In elderly cancer patients in particular, a significantly increased percentage of NK cells among peripheral blood lymphocytes and a raised total number of NK cells has been found [4]. NK cells activity in peripheral blood samples of patients with laryngeal carcinoma seems to have a favorable prognostic effect on survival [5]. Specific therapeutic treatments are reported to affect NK activity having an immunoenhancing effect on the patients [6, 7].

As far as other types of cancer are concerned, Coca et al, using the monoclonal antibody CD57, reported that in patients with colorectal carcinoma, an extensive intratumoral infiltration of NK cells is associated with a favorable tumor outcome [8]. In breast cancer, the prognostic significance of NK cell presence remains questionable [9].

The aim of the present study was to evaluate the distribution of NK cells in tissues of laryngeal carcinomas and explore any possible association of their semiquantative presence with various clinicopathological prognostic variables, including TNM stage, histologic degree of differentiation as well as patients‘ recurrence-free survival.

* Corresponding author

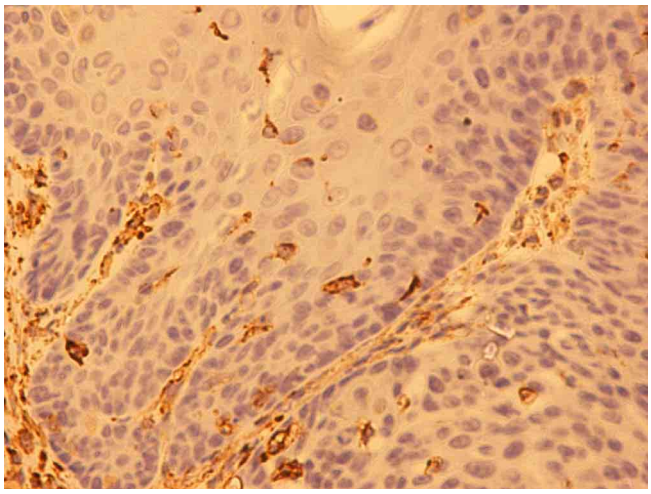

Figure 1. Increased number of CD16-immunoreactive cells in proximity with laryngeal cancerous formations (Immunoperoxidase stain with anti-CD16 antibody, x 200).

Table I. CD16+ cell distribution in relation to patients' clinicopathological variables.

Variable		NK cell presence	
		Low – Intermediate	**High**
1.	**Tumor location**		
	Supraglottic: 8	5	3
	Glottic: 7	6	1
	Transglottic: 16	13	3
2.	**TNM stage**		
	I: 9	8	1
	II: 7	6	1
	III: 4	3	1
	IV: 11	7	4
3.	**Relapse**		
	Yes: 11	9	2
	No: 20	15	5
4.	**Ki67 immunoexpression**		
	Low (≤ 20% of cancer cells): 9	7	2
	High (> 20% of cancer cells): 17	14	3
5.	**p53 protein accumulation**		
	Negative (≤ 10% of cancer cells): 10	8	2
	Positive (> 10% of cancer cells): 18	14	4
6.	**Cathepsin D expression in neoplastic cells**		
	Negative: 20	16	4
	Positive: 9	6	3
7.	**TRF1 expression**		
	Negative: 15	13	2
	Positive: 16	11	5
8.	**Histologic differentiation**		
	High to moderate: 16	15	1
	Poor: 14	8	6

Materials and methods

The study included 30 males and 1 female with previously untreated epidermoid carcinoma of the larynx (mean age, 61.32 years; range, 42-75 years). After mean follow-up of 24.067 months (range, 4-36 months), 11 patients (35.4%) relapsed. The respective tumor sections were re-evaluated with regard to pathological parameters including tumor grade by two independent pathologists. The characteristics of our sample are shown in Table I. Patients' management is illustrated in Table II.

In the present study, NK cells were identified by their immunohistochemical expression by a panel of monoclonal antibodies (against CD16, CD56 and CD11b antigens) on frozen tissue sections from fresh surgical specimens of laryngeal carcinoma. In parafin sections, monoclonal antibodies of NK cells (anti-Leu-7, CD 57) have so far been applied; however, they have been observed to stain large granular lymphocytes and a subject of $CD8^+$ cells as well. On the other hand, it is noteworthy that approximately 20% to 70% of NK cells are reported to express CD57 [1]. The recognition element on NK cells is CD16, the low-affinity Fc receptor for IgG [1]. Other NK cell-associated antigens include CD56 and CD11b. In peripheral blood, the presence and intensity of CD16 and CD56 expression permits division of the NK cell population into two distinct subsets: a) approximately 90% of peripheral blood NK cells express low levels of CD56 and abundant CD16; the latter mediate non-MHC-restricted cytotoxicity b) the remaining 10% of peripheral blood $CD16^{dim/negative}$ $CD56^{bright}$ NK cells do not mediate strong cytolytic activity. Therefore, in our samples, tissue identification of NK cells was finally based on strong CD16 immunostaining and three sections from each tumor were separately stained with the following three primary monoclonal mouse antihuman antibodies, all provided by Dako, Glostrup, Denmark: a) anti-Fc Gamma Receptor II, CD16, clone VIFcRII, isotype IgM, Kappa b) anti-NK cell, CD56, clone T119, isotype IgG1, Kappa and c) anti-CD11b, C3bi Receptor, clone 2LPM19c, isotype IgG1, Kappa. A three-step immunoperoxidase staining procedure was performed. Each of the above mentioned antibodies was diluted 100 times (1:100) and the slides were incubated at 4^o C in a humidity chamber, overnight.

NK cell presence, proved predominantly by CD16 immunoreaction, was scored on a semiquantitave scale (low to intermediate and high) and possible associations were investigated between this presence and various clinicopathological variables (Table I) including patients' relapse and the expression of various immunohistochemical markers of potential prognostic significance in laryngeal cancer [10, 11] (data obtained from our archives database).

When assessing immunopositive staining percentages for each immunomarker, cellular morphology of immunoreactive cells was taken into account. Morphologically, NK cells are somewhat larger than small lymphocytes, and they contain abundant azurophilic granules. Immunoreactive cells were considered as positive only when their morphology was consistent with that described above (Fig. 1). In detail, staining results for each NK cell marker were evaluated as follows: when the percentage of immunopositive cells was less than 5% of the lymphocytes within or around tumor parenchyma,

NK cell presence was graded as low; when the above percentage ranged from 5 to 20%, NK cell presence was graded as intermediate and when the proportion of immunopositive cells among lymphocytic infiltrates exceeded 20%, NK cell presence was characterized as high. Based on the above cut off points, staining results for all three NK cell markers were graded by two independent pathologists under light microscopy of the whole section of each tumor. As far as the semiquantative categorization of the 31 cases is concerned, discordance was detected in only one case concerning CD 11b immunolabelling with percentages of CD11b-immunoreactive cells around 20%; finally, concensus was achieved and the case was added to the group of tumors with high NK cell presence.

With regard to the other immunohistochemical markers the staining results of which were available from previous studies [10, 11], the following antibodies (Abs) had been used: a) rabbit antihuman Ki67 antigen Ab (Dako, Glostrup, Denmark) b) monoclonal mouse antihuman p53 Ab, DO-7 (Dako, Glostrup, Denmark), c) rabbit antihuman cathepsin D Ab (Dako, Glostrup, Denmark) and d) goat polyclonal Ab to telomeric repeat binding factor-1 (TRF-1, SantaCruz Biotechnology, California, USA).

Chi square statistics were used to investigate the association of NK cells expression (low to intermediate vs high) with grade, stage, location, Ki67, p53, cathepsin D and TRF-1 expression. The distribution of age and tumor maximum diameter among NK cell categories was evaluated by analysis of variance. Disease-free distribution curves of NK cell categories were compared by log rank test. Statistical significance was accepted at the 0.05 level. Statistical correlation between labeling for the three antigens was examined by Wilcoxon signed ranks test.

Results

High CD16$^+$ cell presence was detected in 7 (22.6%) carcinomas (Fig. 1) while low and intermediate CD16$^+$ cell presence was noticed in 7 (22.6%) and 17 (54.8%) tumors respectively. The majority of CD16$^+$ cells also expressed one or both of the other NK cell markers; this was evident after comparative evaluation of representative high power fields of the three stained sections for each case by computer based image analysis. The semi-quantitative distribution of the staining results for all three antibodies is shown in Fig. 2. With regard to semi-quantitative staining results, no statistical differences were noticed either between CD16 and CD11b or between CD56 and CD11b (Z=-0.816, p=0.688 and Z=-1.667 and p=0.180 respectively). In contrast, CD56 and CD16 semi-quantitative staining results differed between them (Z=-2.646, p=0.016). CD56 appeared to be a more sensitive marker than CD16.

There was no statistically significant relation of CD16$^+$ cell presence to patients' age (ANOVA, p=0.571), tumor location (p=0.797), tumor maximum diameter (ANOVA, p=0.238), tumor TNM stage I–V (p=0.381), patients' relapse (Pearson chi square, p=0.692), proliferation (Ki67) index (p=0.743), p53 protein accumulation in cancer cells (p=0.679), cathepsin D immunolabeling (p=0.124) and TRF-1 immunohistochemical expression, an indirect potential marker of telomeric activation (p=0.483), as shown in Table I.

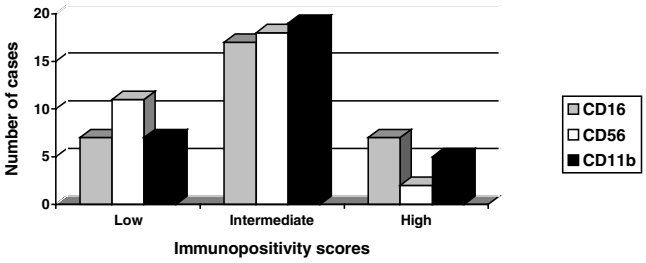

Figure 2. The semi-quantitative scores for the three NK cell markers of the present study.

Table II. Patients' management

	Frequency	Percent
Total laryngectomy	10	32.26
Supraglotic Laryngectomy+ND	3	9.68
Radiation	4	12.90
Total Laryngectomy+Radiation+ND	3	9.68
Total Laryngectomy+ND	5	16.13
Vertical Laryngectomy	2	6.45
Laser+Radiation	1	3.22
Supraglotic Laryngectomy	1	3.22
Laser	2	6.45
Total	31	100.0

Interestingly, high CD16$^+$ cell presence was more often detected in poorly differentiated carcinomas when compared to well and moderately differentiated ones; this difference reached statistical significance (p=0.031). As far as the other two NK cell markers are concerned, no statistically significant differences were noticed in their semi-quantative expression.

With regard to the prognostic impact of CD16$^+$ cell tissue presence, using Kaplan-Meier analysis, this parameter was not related to patients' disease-free survival (log rank, p=0.6635).

Discussion

The underlying basic mechanisms of cancer development, the genes involved, the responsible mutations and the affected proteins, as well as biological markers have been the center of extensive research during the last decade. All these factors may dramatically change the management and prognosis of patients with malignant neoplasms.

Similarly to a previous study on breast cancer [9], in the present series of laryngeal carcinomas, poorly differentiated

cancer cells appear to be more capable of enhancing or altering the target antigens recognized by NK cells than well and moderately differentiated cancer cells. These tumor-specific antigens are derived from mutant forms of normal cellular proteins or may also be generated by gene activation since, during malignant transformation, a mutation may lead to the transcription of a gene that is normally silent. Poorly differentiated, potentially aggressive cancer cells probably bear more genetic alterations than cancer cells of higher histologic degrees of differentiation and so are likely to possess more target antigens. The role of cancer-mesenchymal cell interaction should also be considered as it may be involved in NK cell infiltration to high grade carcinoma tissue. Nevertheless, the efficacy of the immune response against poorly differentiated carcinomas is questionable due to the operative mechanisms which tumor cells are known to develop in order to escape or evade the immune system in immunocompetent hosts. So, the finding of a high number of NK cells in poorly differentiated cancer does not necessarily suggest a favorable outcome.

On the other hand, the value of degree of differentiation, on its own, as an independent prognostic marker in laryngeal cancer is rather questionable.

Gonzalez et al [5] reported that the independent ominous prognostic factors of overall survival in patients with laryngeal carcinoma included histologically determined nodal involvement, low NK cell activity in peripheral blood and tumor grade (the latter though with a borderline statistical significance). The prognostic value of the comparative tissue estimation of functional NK cells infiltrating laryngeal carcinoma warrants investigation in larger series with increased length of follow up which will permit a reliable multivariate statistical analysis. In this way the hypothesis that NK cell activity may play a role in tumor progression or outcome may be reinforced.

The authors would like to express their gratitude to miss Evie Delicha, MSc, Biostatistician, for her valuable contribution to the statistical analysis of the data.

References

[1] KNOWLES DM. Hematopathology. 2nd ed. Philadelphia: Lippincot Williams and Wilkins; 2001

[2] TAKES RP, BAATENBURG DE JONG RJ, et al. Markers for assessment of nodal metastasis in laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1997; 123: 412–419

[3] ZEROMSKI J, DWORACKI G, KRUK-ZAGAJEWSKA A, et al. Assessment of immunophenotype of potentially cytotoxic tumor infiltrating cells in laryngeal carcinoma. Arch Immunol Ther Exp (Warsz) 1993; 41:57–62

[4] DWORACKI G, TRYBUS M, JEZEWSKA E, et al. Immunophenotype of blood lymphocytes in elderly patients with laryngeal cancer. Otolaryngol Pol 1998; 52: 397–400

[5] GONZALEZ FM, VARGAS JA, LOPEZ-CORTIJO C, et al. Prognostic significance of natural killer cell activity in patients with laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1998; 124: 852–856

[6] DENARO A, STIVALA F, MAZZARINO MC. Immunologic study on patients with head and neck cancer treated with thymopentin associated with surgery, chemotherapy and radiotherapy. Acta Otorhinolaryngol Ital 1994; 14: 611–625

[7] LAI JP, TAO ZD, XIAO JY, et al. Microinvasive Nd:YAG laser therapy of early glottic carcinoma and its effect on soluble interleukin-2 receptor, interleukin-2 and natural killer cells. Laryngoscope 2001; 111: 1585–1588

[8] COCA S, PEREZ – PIQUERAS J, MARTINEZ D, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer 1997; 79: 2320–2328

[9] VGENOPOULOS S, LAZARIS AC, MARKOPOULOS C, et al. Immunohistochemical evaluation of immune response in invasive ductal breast cancer of not-otherwise-specified type. Breast 2003; 12: 172–178

[10] LAZARIS ACH, LENDARI I, KAVANTZAS N, et al. Correlation of tumor markers p53, bcl-2 and cathepsin D with clinicopathological features and disease-free survival in laryngeal squamous cell carcinoma. Pathol Int 2000; 50: 717–724

[11] LAZARIS ACH, RIGOPOULOU A, TSELENI-BALAFOUTA S, et al. Immunodetection and clinico-pathological correlates of two tumour growth regulators in laryngeal carcinoma. Histol Histopathol 2002; 17: 131–138