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Cluster of differentiation 8 T-cell population in the laryngeal mucosa of smokers with laryngeal cancer

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Main Article

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Abstract

Objective. To study the cluster of differentiation 8 population in the laryngeal mucosa of patients with laryngeal carcinoma. To our knowledge this is the first paper to address this issue.
Methods. The study group included 40 patients with known laryngeal cancer who were scheduled for laryngectomy. The control groups included 10 smokers and 10 non-smokers who were scheduled for microlaryngeal surgery. Specimens from the three groups were processed for histopathological and histochemical evaluation.
Results. In patients without cancer of the larynx, the number of cluster of differentiation 8 lymphocytes was greater in smokers than non-smokers. The number of cluster of differentiation 8 lymphocytes was greatest in smokers with laryngeal cancer, and the difference between this group and the two control groups was statistically significant.
Conclusion. The study showed that smoking increased the number of cluster of differentiation 8 T-lymphocytes in the laryngeal mucosa. The increase was greatest in patients who had developed laryngeal cancer.

Introduction

Smoking is the largest preventable risk factor for morbidity and mortality in industrialised countries. The World Health Organization estimates that tobacco will become the largest single health problem by 2020, causing an estimated 8.4 million deaths annually (as cited in Vainio *et al.*[1]).

Cigarettes, cigars and pipe tobacco are made from dried tobacco leaves, as well as ingredients added for flavour. More than 4000 different chemicals have been found in tobacco and tobacco smoke. Amongst these are more than 60 chemicals known to cause cancer (carcinogens).[2,3]

Immunophenotyping is a technique used to study the protein expressed by cells. This technique is commonly used in basic science research and laboratories for diagnostic purposes. The cluster of differentiation is a protocol used for the identification and investigation of cell surface molecules providing targets for immunophenotyping of cells.

Two commonly used cluster of differentiation molecules are cluster of differentiation 4 and cluster of differentiation 8. These molecules are generally used as markers for helper and cytotoxic T-cells, respectively. Cluster of differentiation 4 is a co-receptor that assists the T-cell receptor with an antigen-presenting cell. Using its portion that resides inside the T-cell, cluster of differentiation 4 amplifies the signal generated by the T-cell receptor by recruiting an enzyme, known as the tyrosine kinase, which is essential for activating many molecules involved in the signalling cascade of an activated T-cell. Cluster of differentiation 4 cells co-ordinate the overall immune response and help activate cluster of differentiation 8 T-lymphocytes, which attack viruses and tumour cells. Cluster of differentiation 8 T-lymphocytes are often called 'effector' or 'cytotoxic' T-cells because they respond to intracellular pathogens and cancer cells.[4]

Studies on the immunological consequences of tobacco smoking have repeatedly shown changes in the immune response of the laryngeal mucosa. The laryngeal mucosa of current cigarette smokers has shown increased numbers of cluster of differentiation 4 T-cells, and there is an association between older age and greater cluster of differentiation 4 T-cell numbers in both epithelium and lamina propria.[5,6] However, to our knowledge, the cluster of differentiation 8 T-cell population in the laryngeal mucosa of smokers with cancer of the larynx has not been previously studied or reported, despite its documented response to cancer cells. The present study was, therefore, geared at studying the population of cluster of differentiation 8 T-lymphocytes in these patients.

Materials and methods

The study was conducted on 60 patients classified into 3 groups: (1) 40 smokers with known laryngeal cancer who were scheduled for laryngectomy; (2) 10 smokers who

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were scheduled for microlaryngeal surgery for the excision of benign lesions (e.g. vocal nodules); and (3) 10 non-smokers with no nasal symptoms who were scheduled for microlaryngeal surgery for the excision of benign lesions. All patients were recruited from the otolaryngology department between January 2011 and December 2015.

All patients gave written informed consent to participate in the study, after being provided with a detailed explanation of the nature and aim of the research.

At the time of surgery, tiny 1 mm^3 biopsies were carefully taken with micro-cupped forceps from the laryngeal mucosa. Each specimen was de-mounted from the biopsy forceps by a fine needle and kept in a sterile container containing 4 per cent formaldehyde; these were sent for histological and immunohistochemical examination.

Two sets of 4 µmm sections were prepared from each specimen. The first set was stained with haematoxylin and eosin stain. The second set was stained for cluster of differentiation 8 antigen receptors using cluster of differentiation 8 antibody (Dako, Glostrup, Denmark) via an indirect, enzyme-conjugated antibody immunohistochemistry technique on Leica Bond-Max immunostainers. The antibody used was monoclonal mouse anti-human, clone C8/144B, from Dako, at a dilution of 1:100. Dako EnVision™ Flex was used as a visualisation system, in a Dako Autostainer Plus Staining System. The chromogen used was 3,3′-diaminobenzidine (DAB). Positive and negative controls were included in each immunohistochemistry run.

Both sets of slides were coverslipped on a Leica automated coverslipping machine using Pertex® mounting medium. The numbers of cluster of differentiation 8 lymphocytes were counted on a Nikon Eclipse E600 microscope, with a field diameter of 0.35 mm and a field area of approximately 0.1 mm^2. The scoring of cluster of differentiation 8 positive cells was conducted via a visual counting method. The slides with biopsy tissue were viewed and evaluated twice by two pathologists. We used the average method for the present study because of its greater reproducibility.[7] This method entails manually counting the cluster of differentiation 8 cells on three microscopic photographs, taken at the same magnification, from areas with the highest percentages of cluster of differentiation 8 cells, and calculating the average numbers of cells. The two pathologists used the same sets of photographs.

Statistical analysis

Data entry and analysis were performed using SPSS® statistical software version 15. Sample size calculation was based on a power of 0.8 and an alpha level of 0.05. Continuous variables are presented as means ± standard deviations. Mean values were compared using *t*-tests. Pearson's correlation co-efficient was used to test for correlations between variables. A *p*-value of less than 0.05 was considered statistically significant.

Results

The demographic data of the patients, including age, sex and smoking history, are shown in Table 1. The numbers of cluster of differentiation 8 T-lymphocytes in the laryngeal mucosa of the three groups are shown in Table 2.

The number of cluster of differentiation 8 T-lymphocytes in smokers without cancer of the larynx was greater than that in non-smokers (Figures 1 and 2), and the difference was statically significant ($p < 0.05$).

Table 1. Demographic data of the three groups

Parameter	Study group*	Control groups	
		Smokers†	Non-smokers‡
Age range (years)	57–64	39–46	36
Sex (male:female ratio)	36:4	10:0	10:0
Smoking starting age (mean ± SD; years)	25 ± 2	19 ± 2	–
Smoking history (cigarettes per day; range)	28–60	16–39	–

*$n = 40$; †$n = 10$; ‡$n = 10$. SD = standard deviation

Table 2. Comparison between laryngeal mucosa CD8 count in the three groups

Parameter	Study group	Control groups	
		Smokers	Non-smokers
Min.	99	8	0
Max.	187	16	11
Mean	150.20	11.6	3.8
SD	34.986	3.209	4.324
P-value 1		0.019	0.013
P-value 2			0.028

P-value 1 reflects the statistical significance of the difference between the study group and control groups. *P*-value 2 indicates the statistical significance of the difference between the two control groups. CD8 = cluster of differentiation 8; SD = standard deviation

The number of cluster of differentiation 8 cells was markedly increased in the laryngeal mucosa of patients with laryngeal squamous cell carcinoma (Figure 3), and there were statistically significant differences between this group and the two control groups ($p < 0.05$).

There were positive correlations between the number of cluster of differentiation 8 cells in patients with laryngeal cancer and the duration of smoking (Table 3) as well as the number of cigarettes consumed per day (Table 4) (R = 0.9528 and 0.9868 respectively).

Discussion

The present study represents a quantitative investigation of the active expression of cluster of differentiation 8 T-lymphocytes in the human laryngeal mucosa of smokers with known cancer of the larynx. A major component of mucosal defence in mammals is provided by the content of immunologically active cells. Cell types identified in the laryngeal mucosa that are associated with changes in challenge states in adult mammals include macrophages, T- and B-lymphocytes, natural killer cells, and macrophages.[8,9]

The cluster of differentiation 8 co-receptor is predominantly expressed on the surface of cytotoxic T-cells, but can also be found on natural killer cells, cortical thymocytes and dendritic cells. A cytotoxic T-cell (also known as 'TC', cytotoxic T-lymphocyte, 'CTL', T-killer cell, cytolytic T-cell, CD8 T-cell or killer T-cell) is a T-lymphocyte that kills: cancer cells, cells that are infected (particularly with viruses), or cells that are damaged in other ways.[4]

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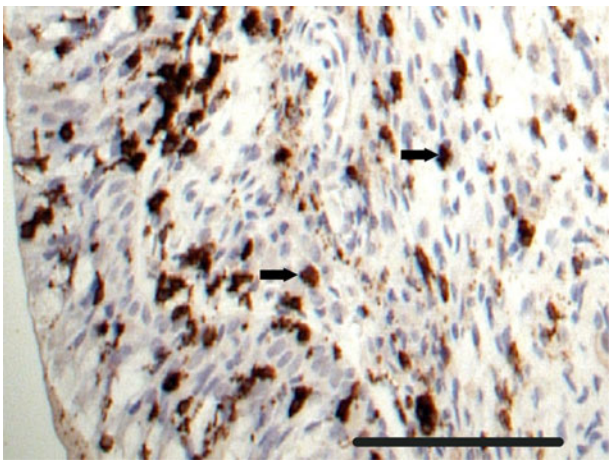

Fig. 1. Cluster of differentiation 8^+ T-cells (black arrows) in the mucosa of smokers without squamous cell carcinoma. (Cluster of differentiation 8 antibody; ×300)

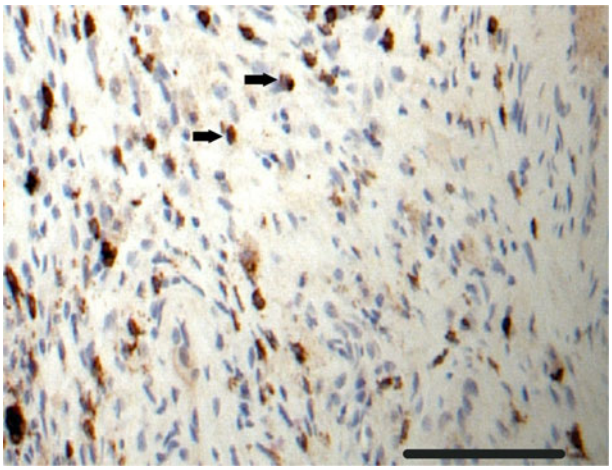

Fig. 2. Cluster of differentiation 8^+ T-cells (black arrows) in the mucosa of non-smokers without laryngeal cancer. (Cluster of differentiation 8 antibody; ×250)

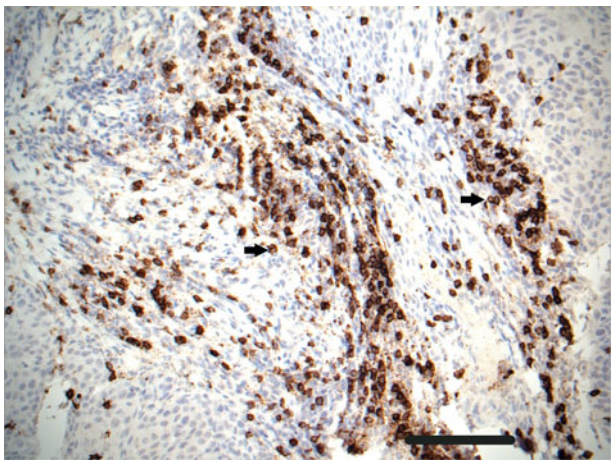

Fig. 3. Cluster of differentiation 8^+ T-cells (black arrows) in the mucosa of smokers with squamous cell carcinoma. (Cluster of differentiation 8 antibody; ×200)

Table 3. Duration of smoking in laryngeal cancer patients and its relation to CD8 cell count

Duration of smoking (years)	CD8 cells (*n*)
10–15	117.5
16–20	131.2
21–25	153.6
25–30	165.1
>30	182.9

R = 0.9528. CD8 = cluster of differentiation 8

Table 4. Number of cigarettes per day in laryngeal cancer patients and its relation to CD8 cell count

Cigarettes per day (*n*)	CD8 cells (*n*)
20–30	117.5
31–40	131.2
41–50	153.6
51–60	165.1

R = 0.9868. CD8 = cluster of differentiation 8

The priming phase of tumour-specific T-cells is thought to involve uptake into endosomes, processing and presentation of tumour antigens by bone-marrow-derived dendritic cells. Dendritic cells move to draining lymph nodes, where they present antigens on major histocompatibility complex class I and class II to cluster of differentiation 8 and cluster of differentiation 4 T-cells, respectively. Full activation of dendritic cells to activate cluster of differentiation 8 T-cells requires cluster of differentiation 4 cell help, via interactions between cluster of differentiation 40 and cluster of differentiation 40-ligand ('CD40L').

Previously published data have shown that cigarette smokers have increased numbers of cluster of differentiation 4^+ T-cells, and there is an association between older age and greater cluster of differentiation 4^+ T-cell numbers in both epithelium and lamina propria. Older age and female gender were associated with decreased lamina propria cluster of differentiation 4^+ $CD45RO^+$ T-cells and an increase in cluster of differentiation 4^+ $CD45RO^-$ T-cells.[5] The authors concluded that smoking induces changes in the normal immunological function of the larynx, which may contribute to the aetiology of inflammatory disease and cancer.

In the present study, we found, for the first time, that current smokers had greater numbers of cluster of differentiation 8 T-cells in the laryngeal mucosa than non-smokers. The number of cluster of differentiation 8 T-lymphocytes in smokers with cancer of the larynx was significantly greater than that in patients without cancer of the larynx regardless of whether they were smokers or non-smokers.

Cluster of differentiation 8 T-lymphocytes are thought to be stimulated by viral antigens and neoplastic changes in the cell, and have a cytotoxic effect on tumour cells. The present study showed that smoking alone may increase the number of cluster of differentiation 8 T-lymphocytes in the mucosa. This was indicated by the increased number of cluster of differentiation 8 T-lymphocytes in smokers without cancer of the larynx compared to non-smokers. This increase was presumably due to the irritant effects of the various noxious molecules in the smoke.

The findings of this study may have implications for the early diagnosis of laryngeal cancer, as they indicate that the increased number of cluster of differentiation 8^+ cells may be a defensive mechanism against a starting or ongoing neoplastic transformation. This may be clinically useful in following up high-risk patients, including heavy smokers, males aged over

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60 years, consumers of large amounts of alcohol, and those with long-term exposure to certain chemicals, fumes or pollutants. The findings may also help in predicting the disease prognosis.[10,11]

This study opens up a number of avenues for further research. First, there is a need to develop normative data for the cluster of differentiation 8 T-lymphocytes count in the nasal, laryngeal and tracheal, and bronchial mucosa of the population, including data related to age, sex and geographical area. Second, there is a need to repeat the study in patients with nasal or bronchial cancer to see if similar findings occur. It would be interesting to find out if the cluster of differentiation 8 counts in these sites rise exponentially with the occurrence of malignancy, as in patients with laryngeal cancer.

Adoptive cell based immunotherapy was first introduced by Rosenberg and colleagues of the National Institutes of Health, USA, and it is now widely used in various countries.[12,13] It involves isolation of either allogenic or autologous immune cells, enriching them outside the body, and transfusing them back to the patient. The injected immune cells are highly cytotoxic to the cancer cells, thereby helping to fight the cancer cells. This therapy is in routine clinical practice in Japan. Autologous immune enhancement therapy involving natural killer cells and cytotoxic T-lymphocytes is also being practised in various Asian countries, especially in Japan and Malaysia.[14] The present study, therefore, may also open the door for investigating the use of adoptive immunotherapy in select patients.

- Smoking increases the number of cluster of differentiation 8 T-lymphocytes in laryngeal mucosa
- The increase in cluster of differentiation 8 counts was greatest in patients who had developed laryngeal cancer
- This increase may be due to the irritant effects of various noxious molecules in the smoke and is presumably a defensive mechanism
- This finding may have implications for early diagnosis of laryngeal cancer or in applying adoptive cell based immunotherapy to smokers with laryngeal cancer

In the present exploratory study, we did not assess cluster of differentiation 4 cells and we did not include other areas of the respiratory tract that might be affected by smoking, including the nose, trachea and bronchi. These are obvious limitations that we intend to address in a forthcoming study.

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Competing interests. None declared.

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