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Major histocompatibility complex class I expression in human tonsillar and laryngeal epithelium

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Summary

Understanding the immunological structure of the upper aerodigestive tract is important for analysing the interaction between incident challenges, such as human papillomavirus infection, and disease, particularly head and neck cancer. We have shown previously that tonsillar and laryngeal epithelium express major histocompatibility complex (MHC) class II locus products, but that expression of human leucocyte antigen (HLA)-DQ is reduced compared to HLA-DR. This may confer a decreased repertoire of presented T cell epitopes generated by the processing of exogenous peptides in upper airway mucosa. To determine whether the peptide repertoire presented by MHC class I loci varies in stratified squamous epithelium, laryngeal and tonsillar biopsies were taken from 19 otherwise healthy patients (M : F 6 : 13, 16–64 years). Quantitative immunofluorescence microscopy, using antibodies to MHC class I α-chain (pan-locus specific, HLA-A, HLA-B + C) and β_2-microglobulin, showed lower expression of the α-chain in laryngeal and tonsillar epithelium than in either lamina propria (tonsil 73% *versus* 89%, $P < 0{\cdot}0001$; larynx 68% *versus* 85%, $P < 0{\cdot}005$). Within the epithelium itself, the intensity of α-chain expression decreased from the basal to apical layers. In paired squamous epithelia from the two sites, α-chain expression was significantly higher in the tonsil compared to the larynx (79% *versus* 62%, $P < 0{\cdot}05$). We suggest that these findings reflect functional stratification of these epithelia with the superficial layer, most exposed to incident challenges, less equipped to present antigens to conventional T cells. This may affect immunosurveillance directed at viral and tumour-related epitopes in the upper airway.

Keywords: epithelial cells, larynx, MHC class I, tonsil

Introduction

The upper aerodigestive tract is exposed to a wide variety of incident challenges, some of which are associated with subsequent cancer development: tobacco [1], alcohol [2], viruses [3] and less conclusively, refluxate [4]. There is good evidence of an association between viral infection and cancer at various subsites within the head and neck, specifically human papillomavirus (HPV) and squamous cell carcinoma of the oropharynx (especially the tonsil) [5] and Epstein–Barr virus and nasopharyngeal cancer [6]. Understanding mucosal immune responses to viral antigens, and the interaction between these factors and other known carcinogens such as tobacco and alcohol, is crucial for developing disease prevention strategies and in considering novel therapeutic approaches such as vaccination.

The major histocompatibility complex (MHC) interacts with T lymphocytes by presenting antigens on the cell surface for recognition. MHC class I molecules are increasingly thought to be important in the aetiology of head and neck carcinogenesis, as they can present endogenous tumour and viral antigens. We have shown previously that human tonsillar and laryngeal epithelial cells constitutively express MHC class II antigens on their surface [7,8]. Importantly, they appear not to express all three human leucocyte antigen (HLA) locus products concordantly (HLA-DP, -DQ and -DR), in that epithelial expression of HLA-DQ is reduced by comparison with intraepithelial leucocytes and lamina propria leucocytes. Because MHC class II is critical for recognition of novel antigenic peptides by $CD4^+$ T cells, differential expression of loci is likely to restrict the potential repertoire presented by epithelial cells. This limited class II repertoire

may confer decreased immunological responsiveness to T cell epitopes generated by processing of exogenous peptides within upper airway mucosa. In addition, the absence of conventional co-stimulatory molecules from epithelial cells may mean that presentation of such antigens leads to tolerance rather than $CD4^+$ T cell activation [9].

It is not known whether expression of MHC class I locus products (HLA-A, -B and -C) is similarly discordant on laryngeal and tonsillar epithelium. If so, selective MHC class I locus expression could similarly restrict immunological responsiveness to viral and tumour-associated antigens, providing important insights into the site and type prevalence of squamous cell carcinoma (SCC) in these areas [10]. The aim of this study was to measure MHC class I expression in laryngeal and tonsillar squamous epithelium and lamina propria, and to explore the variability between individuals.

Materials and methods

Design

This was a multi-centre, hospital-based, cross-sectional biopsy study.

Ethical approval

Ethical permission was obtained from the local research ethics committees at North Bristol NHS (National Health Service) Trust (139/02) and United Bristol Hospitals NHS Trust (E5540), Bristol, UK. Informed written consent was obtained from all individuals prior to commencement of the study.

Population

Tonsils were obtained from individuals undergoing tonsillectomy for recurrent acute tonsillitis, quinsy or during surgery for snoring or obstructive sleep apnoea (Table 1). Pinch biopsies of supraglottic mucosa were obtained from these subjects with additional consent. Patients with a past or current history of malignancy were excluded.

Table 1. Demographic characteristics of patients.

Characteristics	Tonsil biopsies $n = 15$ (%)	Laryngeal biopsies $n = 9$ (%)
Median age (years)	22	19
Age range (years)	17–64	16–41
Gender		
Male	5 (33%)	3 M (33%)
Female	10 (67%)	6 F (67%)
Current smokers	6 (40%)	7 (78%)
Ex-smokers	1 (7%)	1 (11%)
Alcohol drinkers	7 (47%)	6 (67%)
Reflux symptoms	3 (20%)	1 (11%)
Allergy to inhalants	4 (27%)	2 (22%)
Occupational carcinogen exposure	5 (33%)	4 (44%)

Tissue storage and preparation

Tonsils were cut in half using a sterile blade and placed onto cork disks before being embedded in optimal cutting temperature compound (OCT) (Tissue-Tek®, Sakura Finetek, Torrance, CA, USA) and snap-frozen in isopentane precooled over liquid nitrogen. Supraglottic biopsies were orientated with the luminal surface outermost and wrapped around a core of OCT previously frozen in liquid nitrogen, before being snap-frozen as above. The specimens were stored at −70°C.

Quantitative multiple colour immunofluorescence microscopy

We have previously validated a method of quantitative immunofluorescence microscopy against manual cell counting [11] and flow cytometric analysis [7]. Briefly, fixed air-dried tissue sections were first blocked with 50 µl per section of 5% human serum and 5% goat serum in phosphate-buffered saline (PBS), washed and stained with 50 µl of primary mouse anti-human monoclonal antibody diluted in PBS in a humidity chamber at 4°C. The following primary antibodies were used: human leucocyte antigen (HLA)-A, B + C (clone W6/32, IgG_{2a} κ; DAKO, Carpinteria, CA, USA); HLA-A (clone 108–2C5, IgG_1 κ; Lab Vision Corp., Fremont, CA, USA); β_2-microglobulin (clone TÜ99, IgM κ; BD Biosciences, San Diego, CA, USA); HLA-B + C (clone TÜ149, IgG_{2a} κ; a kind gift from Professor A. Ziegler, University of Berlin, Germany) and common leucocyte antigen, CD45 (clone HI30, IgG_1 κ; BD Biosciences). All concentrations were optimized by serial dilution analysis on sections from control tonsil specimens. Two negative control sections (one without primary antibody and one without secondary antibody) were used for each specimen.

After incubation and washing, 50 µl of optimal dilutions of isotype-specific goat anti-mouse monoclonals conjugated with fluorochrome or biotin were added (Southern Biotechnology Associates Inc., Birmingham, AL, USA). Where biotinylated secondary antibody was used, a tertiary step with 50 µl of 1% aminomethylcoumarin (AMCA) avidin diluted in PBS (AMCA avidin D, Vector Laboratories Inc., Burlingame, CA, USA) was required for a further 1-h incubation at room temperature. When a nuclear stain was necessary, 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Corporation, St. Louis, MO, USA) was diluted at 0·08 mg/ml in PBS, and added at 50 µl per section for 10 min. Finally, the sections were washed in PBS and mounted using Vectashield (Vector Laboratories Inc., Burlingame, CA, USA).

Microscopy was performed on multiple sections at 20× and 40× magnification using a Leica DMR fluorescence microscope (Leica, Wetzlar, Germany) with a combined red, green and blue filter. Images were captured and digitized

using a high sensitivity colour camera (Colour Coolview, Photonic Science, UK) and Image ProPlus software (Media Cybernetics, Silver Spring, MD, USA).

Image analysis

Area analysis

Analysis was performed using ImageJ software as described [11] (available in the public domain from http://rsb.info.nih.gov/ij/). Ten images in total were analysed for each sample (five at each magnification) with an area of interest (AOI) being drawn around the epithelium and lamina propria as appropriate. Positive staining was then expressed as a proportion of total pixels.

Line analysis

Line analysis was undertaken using a commercial image processing package (Image ProPlus, Media Cybernetics, Silver Spring, MD, USA). The individual intensity of red, green and blue pixels along a chosen line was used to profile intensity of expression of target molecules across the epithelium from basal to apical layers. Starting at the basement membrane, five perpendicular lines were drawn on each 20× field so that they reached the outermost part of the epithelium. A control line was drawn on a part of the field which contained no tissue to obtain a background intensity and intensities of red, green and blue fluorescence along test lines were normalized to minimum of the background intensity and maximum of peak intensity along the line. Each line was split into 10 equal segments between basement membrane and luminal surface and the average intensity of each decile calculated to allow comparison of lines of different length. The information from five fields per individual was averaged.

Statistical analysis

Software used was SPSS version 12·0 for Windows (SPSS Inc., Chicago, IL, USA). Paired *t*-tests were used to compare intensities of antigen expression between epithelium and lamina propria and between laryngeal and tonsillar sites in each individual. Where standard deviations were unequal, central tendency was expressed using a geometric mean and a paired *t*-test was performed on $\log_{10}$-transformed data. Significance was accepted at the 5% level.

Results

Clinical and demographic characteristics of patients

Biopsies from 19 individuals were analysed (10 tonsil samples, four laryngeal samples and five paired samples of both tonsil and laryngeal tissue). The baseline characteristics of the groups were broadly similar (Table 1).

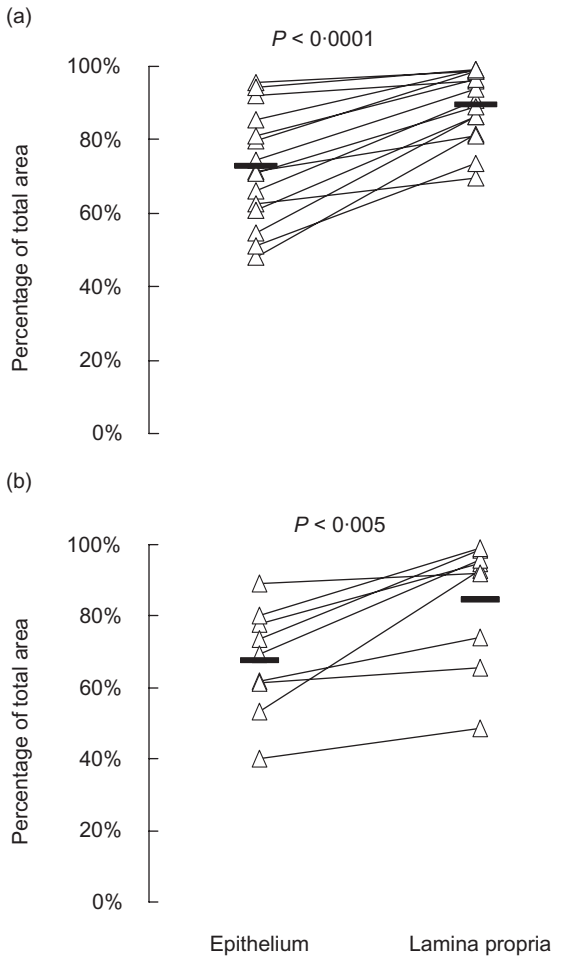

Fig. 1. Total major histocompatibility complex (MHC) class I expression (positive area as a percentage of total area) in (a), tonsil epithelium and (b), laryngeal epithelium compared to underlying lamina propria. Both sites expressed significantly lower proportions of MHC class I α-chain in the epithelium and demonstrated high intersubject variability.

MHC class I expression

The proportion of the total area of epithelium and lamina propria which expressed MHC class I was highly variable between individuals at both sites. Despite the interindividual variation, tonsil and laryngeal epithelia in any single individual expressed significantly lower amounts of total MHC class I α-chain compared to the underlying lamina propria in the same individual (Fig. 1). In the epithelium, the intensity of MHC class I expression decreased from high levels at the basement membrane to low levels in the apical layers, while $CD45^+$ cells (intraepithelial leucocytes) were scattered throughout the epithelium, decreasing in numbers nearer the luminal surface but often present with very little surrounding epithelial cell MHC class I (Fig. 2b). A DAPI stain confirmed intact nuclei in the cells of the apical layers, without obvious signs of pyknosis or apoptosis (Fig. 2c).

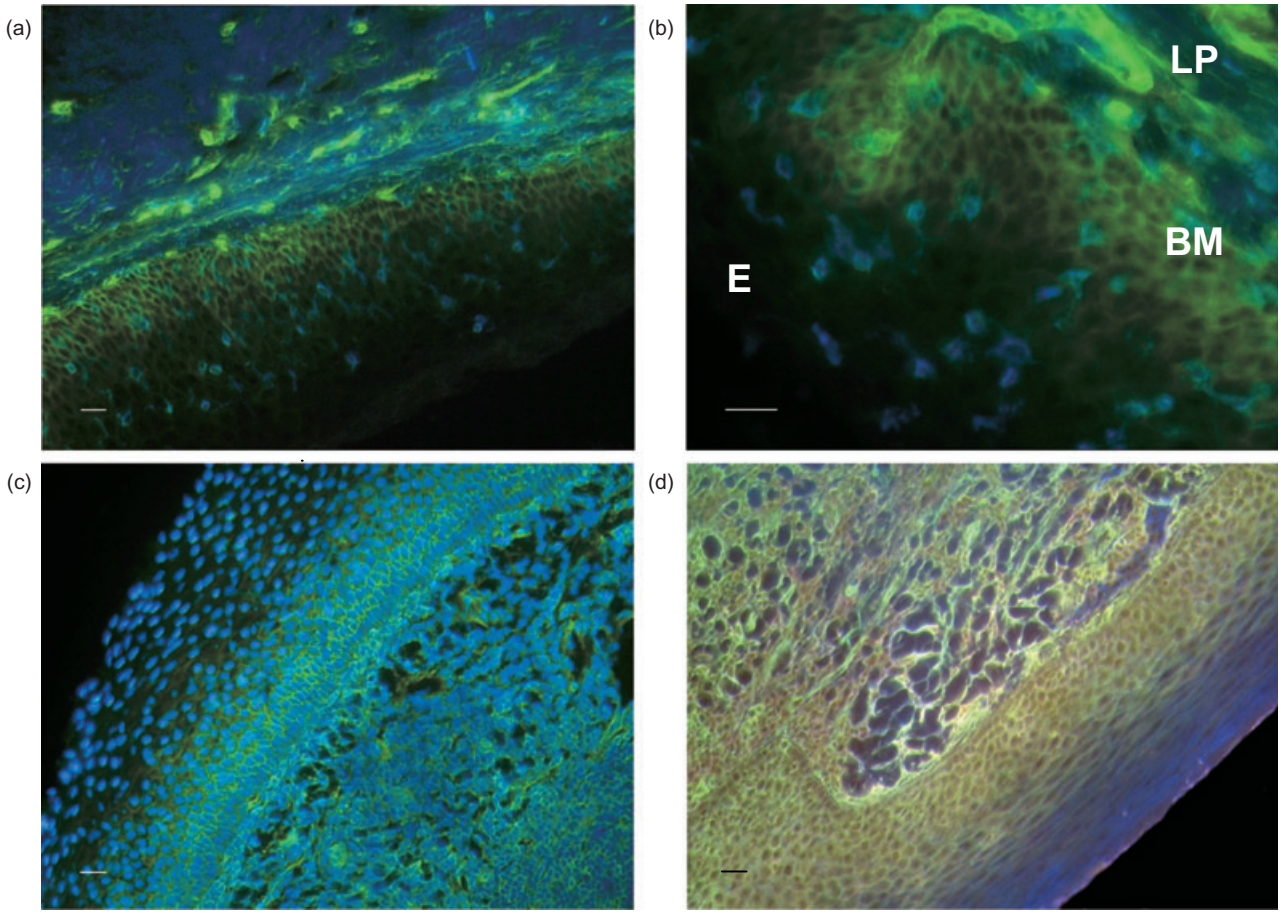

Fig. 2. (a) Major histocompatibility complex (MHC) class I expression in a laryngeal biopsy from a 21-year-old-female (MHC class I, green; CD45, blue, 50 μm scale bar). (b) Higher magnification of the same biopsy (basement membrane, BM; lamina propria, LP; squamous epithelium, E). (c) MHC class I expression in tonsillar epithelium with a 4,6-diamidino-2-phenylindole (DAPI) stain showing condensed but intact nuclei at the luminal surface (MHC class I, green; DAPI nuclear stain, blue). (d) MHC class I locus expression in a tonsillar biopsy from a 25-year-old-female (HLA-A, green; HLA-B and -C, red; β_2-microglobulin, blue). HLA-A, -B and -C are largely co-localized (orange–yellow). There is a decrease in intensity of MHC class I towards the lumen, while β_2-microglobulin appears to persist.

In the lamina propria, MHC class I expression was variable depending on the extent of connective tissue present. This tissue tended to autofluoresce with a blue wavelength. General autofluorescence was accounted for in the analysis of negatively stained specimens and subsequent background threshold setting, while specific areas of autofluorescence were not included in areas of interest in the image analysis.

Expression of different MHC class I loci

The patterns of expression of MHC class I locus products were similar at both mucosal sites. HLA-A, -B and -C and β_2-microglobulin were expressed at significantly lower levels in the epithelium compared to the underlying lamina propria (Fig. 3a,f). As with pan-MHC class I expression, the intensity of HLA-A, -B and -C decreased from high levels at the basement membrane to low levels in the apical layers. The rate of decrease in expression was similar across both epithelia. In contrast, expression of β_2-microglobulin decreased less, remaining more prominent in the apical layers (Fig. 2d), as demonstrated by the line analysis (Fig. 4).

Paired tonsil and laryngeal epithelia

When paired samples of tonsil and laryngeal epithelium from the same individual were compared, α-chain expression was found to be significantly higher in the tonsil (79% *versus* 62%, $P = 0{\cdot}048$) (Fig. 5a). Using locus-specific staining, differences in HLA-A between tonsil and larynx were significant ($P = 0{\cdot}012$) (Fig. 5b), but not HLA-B and -C ($P = 0{\cdot}065$) or β_2-microglobulin ($P = 0{\cdot}236$) (data not shown).

Discussion

This study has four major findings. First, MHC class I expression was significantly lower in the epithelium of the human tonsil and larynx compared to the underlying

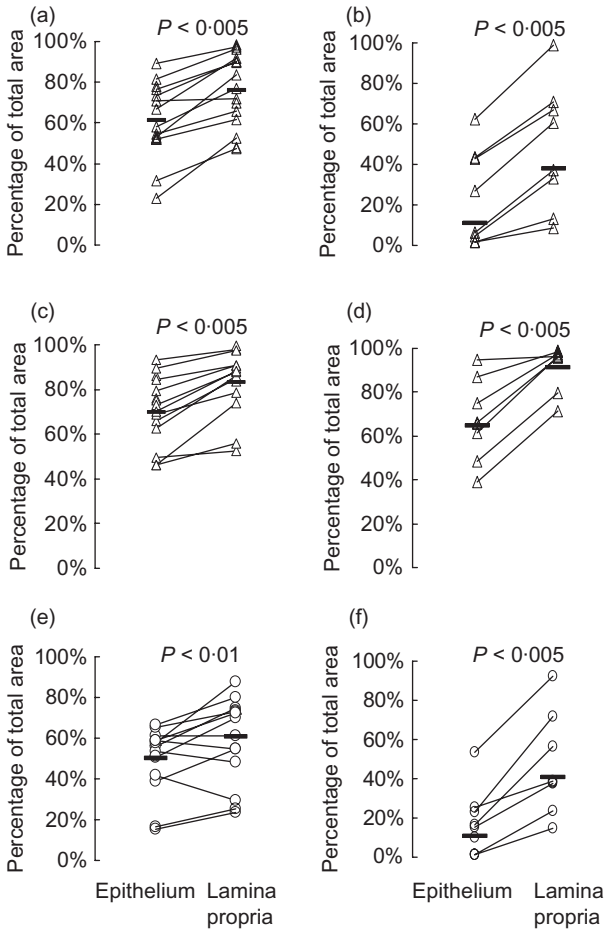

Fig. 3. Major histocompatibility complex (MHC) class I locus expression showing decreased expression in tonsil epithelium [(a), HLA-A; (c), HLA-B and -C; (e), β_2-microglobulin] and laryngeal epithelium [(b), HLA-A; (d), HLA-B and -C; (f), β_2-microglobulin] compared to underlying lamina propria.

lamina propria. Secondly, MHC class I expression was significantly lower in the apical layers of the epithelium compared to the basal layers. Thirdly, in paired squamous epithelia from cancer-free subjects, MHC α-chain expression appeared to be significantly higher in the tonsil compared to the larynx. Finally, MHC class I expression at these two mucosal sites was highly variable between different individuals.

MHC class I molecules are expressed on most nucleated cells in the body, although their levels can vary widely depending on the tissue [12]. For example, the cells of the immune system express relatively higher levels of MHC class I than other cell types [13]. However, previous studies have used qualitative methods of immunohistology. This present study provides quantitative assessment of MHC class I expression *in vivo* and is therefore able to identify subtle differences in expression either between different microenvironments in a single microscopic field or between different mucosal sites. Our studies indicate three such microenvironments based on MHC class I expression: mucosal lamina propria, basal epithelium and apical epithelium. Differential expression of MHC class I in these sites may have implications for immunosurveillance directed at viral and tumour-related epitopes.

We hypothesize that lower levels of MHC class I in the epithelium of the tonsil and larynx are likely to result in a reduction in the ability to present endogenous, MHC class I-restricted peptides. By implication, the differences in expression between the apical and basal layers of the epithelium means that the former will be less subject to MHC class I-restricted/CD8 cytotoxic T cell-mediated immunological surveillance, despite the likelihood of a higher level of exposure to potential mutagens. The finding that expression of β_2-microglobulin did not decrease as much as α-chain in the apical layers may indicate a reliance on MHC class IB/natural killer (NK) cell-mediated surveillance. Contenders for a β_2-microglobulin-associated MHC class IB molecule include HLA-E or CD1d, although CD1d can also be β_2-microglobulin-independent. In support of this, both are expressed on intestinal epithelial cells (IECs) [14], and we have performed

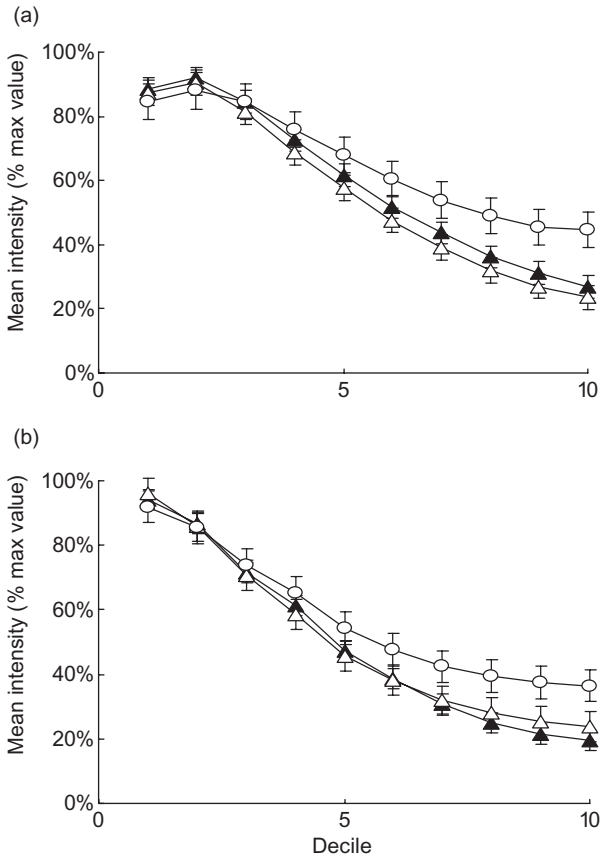

Fig. 4. Analysis of intensity of expression of major histocompatibility complex (MHC) class I loci in (a), tonsil and (b), larynx epithelium (△, HLA-A; ▲, HLA-B and -C; ○, β_2-microglobulin) showing similar rates of decrease in MHC α-chain expression, but not β_2-microglobulin, which remained more prominent in the apical layers.

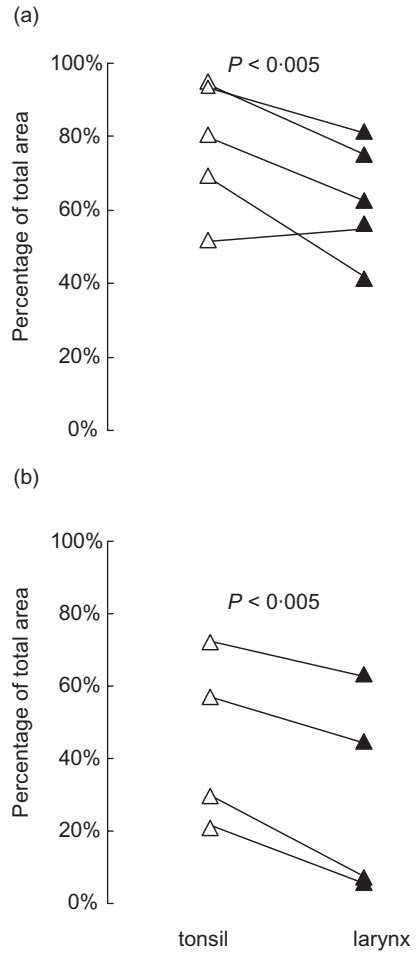

Fig. 5. (a) Total major histocompatibility complex (MHC) class I and (b) HLA-A expression in paired epithelia showing lower levels in the larynx.

a pilot study which confirms the expression of both CD1d and CD161 (NK/NK T) within laryngeal epithelium (data not shown).

MHC class I loss or down-regulation is a major mechanism by which both viruses and tumours can evade cytotoxic T cell responses [15]. Specifically, this has been demonstrated in several studies of laryngeal squamous cell carcinoma [15–17]. It is therefore interesting to note the change in MHC class I expression from basal to luminal across the epithelium and to hypothesize that neoplastic change in the superficial layers of epithelial cells may be less susceptible to immune control, as they appear to have constitutively down-regulated MHC class I.

With specific respect to the potentially oncogenic effects of HPV [5], it has been observed that HPV sequesters itself in the outer layers of the epithelium: although it infects primitive basal cells, viral replication and assembly are confined to differentiating superficial epithelial cells [18]. Although it has been shown that the E5 protein of HPV can down-regulate MHC class I expression [19,20] HPV may, in addition, be exploiting the underlying differences in epithelial MHC class I expression observed here.

The finding that MHC α-chain expression was significantly higher in the tonsil compared to the larynx was surprising and is possibly related to the local cytokine environment (particularly interferons). Importantly, the lower expression of class I by the laryngeal epithelium may, similarly, explain why HPV-related papillomas and squamous cancer are more common in the larynx than in the tonsils [21].

The variability in MHC class I expression between individuals may also help to explain predisposition to malignant transformation. The observation that individuals with lower levels of MHC I in larynx also demonstrated low levels in tonsil indicates that this is not a sampling error and that it reflects factors common to more than one site, although changes over time were not addressed in this study. It is probable that some predisposition for development of head and neck cancer exists, because not all individuals who are exposed to carcinogens such as tobacco go on to develop cancer, but it is not clear to what extent this tendency is inherited or whether this may be a result of exposure to further, undefined environmental components. Studies have confirmed specific HLA–allele associations with head and neck cancer [22] and there is a weak familial link [23], thereby implying at least some genetic influence. However, a prospective longitudinal study would be required to confirm whether low MHC class I is robust over time, and whether level of expression translates into increase risk of cancer. Mechanistic studies aimed at understanding the biological consequences of these changes are also required, as are further observational studies to explore the interaction between MHC class I expression (both classical and non-classical) and exposure to potential environmental carcinogens such as tobacco.

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