Original Article

HPV-induced recurrent laryngeal papillomatosis: fatty acid role-players

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Human papilloma virus-induced recurrent laryngeal papillomatosis is considered a troublesome and dangerous disease, because it can cause airway obstruction. Better understanding of metabolic pathways followed under pathological conditions can contribute to improved therapies by which growth and recurrence may be obviated. Part I of this study presents a clinically relevant total lipid fatty acid profile for papilloma cells, analyzed by gas liquid chromatography and a phosholipid red blood cell profile for RLP patients, analyzed by thin layer chromatography. In the papilloma cells virus interference with delta-6 and delta 5- desaturase activities is prevalent and the n–9 FA metabolic pathway is followed. It is plausible that up-regulated fatty acid synthase and Δ^9 desaturase activities occur, since enhanced saturated fatty acids and monounsaturated fatty acid levels are also prevalent. High saturated fatty acid levels are known for their propensity to interfere with delta-6 and delta-5 desaturase activities and this is reflected in the blood profile of the RLP patients. It is also known that enhanced saturated fatty acid levels can contribute to enhanced cyclooxygenase-2 activity. Furthermore, cumulative oxidative stress with an oxidative burst is responsible for complete exhaustion of exogenous dietary arachidonic acid intake in these patients. The role of linoleic acid needs to be defined. The dietary intakes of lipids and micronutrients in RLP patients and a rationale for adjuvant FA therapy in the management of these patients are discussed in parts II and III of the study.

Key Words: Human papilloma virus, recurrent laryngeal papillomatosis, fatty acid role- players

INTRODUCTION

Recurrent laryngeal papillomatosis (RLP) is a disease characterized by benign squamous epithelial tumors of the larynx that often express aggressive growth, but seldom become malignant. It is mostly caused by human papilloma virus (HPV) types 6 and 11 and often occurs among children. The mode of transmission is still subject to controversy, but a theory of vertical transmission describes the transfer of the virus from the mother infected with *condyloma acuminata* to offspring. RLP are most frequently encountered in the vestibule of the larynx and on vocal cords from where they may expand and spread. Clinically, patients present with progressive hoarseness, stridor and respiratory distress. RLP can regress spontaneously or may have a severe and unrelenting course that can cause significant morbidity and even mortality if left untreated. Currently, the mainstay of treatment for RLP is surgical debulking (laser therapy and removal with a microdebrider or forceps) in combination with adjuvant antiviral drugs.[1, 2]

During essential fatty acid (EFA) metabolism, linoleic acid (LA) and α-linolenic acid (αLA) are converted to longer chain fatty acids (FAs) by steps that include delta-6 and delta-5 desaturase (Δ^6d and Δ^5d) activities. Viruses, saturated fatty acids (SFAs) and *trans*-FAs may interfere with these desaturase activities.[3,4] It is reported that a lack of Δ^6d and Δ^5d activities may occur at different steps during FA metabolism and that their activities may change in concert with alterations in nutritional and hormonal status.[5,6] HPV interference with essential fatty acid (EFA) metabolism may be a serious condition that can hamper the clinical course of RLP management and therefore needs attention.

A comprehensive study on recurrent laryngeal papillomatosis was conducted and in this paper papilloma and red blood cell FA profiles are addressed in an attempt to identify FA role-players in this disease.

SUBJECTS AND METHODS

Black South African patients (n=10), both sexes and between 4 and 12 years of age, were selected. Tumor biopsies (n=10) were taken from papilloma growths within the laryngeal vestibule or on the vocal cords. Control biopsies were taken from normal mucosa of the same patient (n=10) (control A), as well as the anterior tonsillar pillar of tonsillectomy patients (n=10) (control B). Ages of the tumor group and control group B were individually matched. Blood samples were collected into a tube pretreated with ethylene diamine tetra-acetic acid (EDTA) as an anticoagulant and

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then centrifuged at 2,000 rpm for ten minutes to isolate the red blood cells (RBCs). All materials were stored at -70°C until used. Ethical approval and parent or guardian consent were obtained.

All cells were extracted with minimal volumes chloroform: methanol (2:1, v/v) and filtered with Whatman no 1 filter paper. Extracts were dried and dissolved in 100 µl chloroform. Total lipid extraction from the papilloma cells was performed, according to the method of Folch *et al.* (1957).[7] The extracts were methylated by the addition of 100µl trimethyl sulphonium hydroxide, according to the method of Butte (1983).[8] FA methyl esters were analyzed on a Hewlett Packard gas chromatograph (model no. 5890) equipped with a Supelcowax 10 polar column (30m x 0.53mm). Nitrogen at a flow rate of 4 ml. min^{-1} was used as carrier gas at a split ratio of 50:1. The inlet temperature was 180°C and the initial column temperature was 145°C, which was increased by 3°C.min^{-1} to a final temperature of 240°C. The peaks were detected with a flame ionization detector at 300°C. *Cis*- FAs were identified by reference to authentic standards.

In the case of RBCs, the extracted lipids were fractionated following its application to a column (140mm x 29mm) of activated (heated overnight at 110°C) silicic acid (200 mesh) (Aldrich). Neutral lipids and phospholipids (PLs) were eluded by successive application of organic solvents, according to the procedure of Kock *et al.* (1993).[9] The lipid fractions were dissolved in diethyl ether and transferred to pre-weighed vials. The weight of each vial was measured after the samples were dried to a constant weight in a vacuum oven at 50°C over P_2O_5. The FA compositions of the total PL class were determined, according to the method and procedure mentioned for TLs. All lipid analyses were performed by the Lipid Biotechnology Group, Department of Microbial, Biochemical and Food Biotechnology, Faculty of Sciences, University of the Free State.

Results were expressed as percentages for comparison with other studies. Descriptive statistics, namely frequencies and percentages for categorical data and means and medians for continuous data, were calculated per group. To compare groups within the same patient statistically, the paired student-*t* test and signed rank test for parametric or non-parametric data were used and 95% confidence intervals (95% CI) for the mean or median differences were calculated. In the case of comparison between patients and healthy controls the student t-test and Mann-Whitney test were performed.

RESULTS

Although control mucosal cells may appear normal, control group A (patients with viral expression) may have

Table 1a. Total fatty acid compositions of tumor cells compared with control groups A and B.

FA	Tumor vs. control A				Tumor vs. control B		
	Med T	Med A	*p*	95% CI	Med B	*p*	95% CI
14:0	0.93	0.0	0.16	-0.19; 0.98	0.00	0.06	0; 1.15
16:0	16.0	17.3	0.25	-4. 8; 2. 45	9.01	0.57	-12.7; 8.89
16:1n–7	4.31	0.0	0.18	0; 4.31	54.4	0.25	-58.6; 0.73
18:0	30.4	31.4	1.0	-11.8; 8.09	18.3	0.43	-13.7; 18.8
18:1n–9	5.16	10.4	0.30	-11.8; 8.17	4.36	0.73	- 6.77; 15.1
18:2n–6	8.60	7.44	1.00	-5.26; 4.66	1.95	0.25	-6.42; 0.42
18:3n–3	0.25	0.0	0.44	0; 0.49	0.00	0.44	0; 0.49
20:2n–6	5.78	2.63	0.08	- 0.52; 5.78	0.00	0.02	2.89; 13.6
20:5n–3	0.40	0.0	0.02	0; 0.53	0.00	0.02	0.40; 0.53
21:0	3.86	0.0	0.04	0; 5.37	0.00	0.01	3.86; 9.55
22:6n–3	0.83	0.0	0.02	0; 0.92	0.00	0.02	0; 0.92

Abbreviations: FA: fatty acid; T: tumor cells; Med: median; 95% CI: 95% confidence interval. Tumor cells: recurrent laryngeal papillomatosis (RLP). Control A: normal mucosal cells from the larynx in the same RLP patient. Control B: normal mucosal cells from the anterior tonsillar pillar of tonsillectomy patients. *Fatty acids:*14:0 (myristic acid); 16:0 (palmitic acid); 16:1n–9 (palmitoleic acid); 18:0 (stearic acid); 18:1n–9 (oleic acid); 18:2n–6 (linoleic acid); 18:3 (α-linolenic acid); 20:2 (eicosadienoic acid); 20:4n–6 (arachidonic acid); 20:5n–3 (eicosapentaenoic acid); 21:0 (heneicosanoic acid); 22:6n–3 (docosahexaenoic acid).

Table 1b. Total fatty acid compositions of tumor cells compared with control groups A and B.

FA groups	Tumor vs. control A				Tumor vs. control B		
	Med T	Med A	*p*	95% CI	Med B	*p*	95% CI
SFA	56.9	61.0	1.0	-15.1; 15.5	41.2	0.20	-3.93; 20.2
MUFA	11.2	11.4	0.50	-14.4; 5.52	56.2	0.13	-6.77; -49.1
PUFA	19.9	12.8	0.16	-3.4; 10.1	1.95	0.02	0; 20.8
PUFAn–3	1.55	0.0	0.02	0.58; 1.85	0.0	0.02	0;1.85
PUFAn–6	18.1	11.9	0.30	-3.34; 8.41	1.95	0.04	-1.42; 19.3

Abbreviations: FA: fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; n–6: omega–6 fatty acid series; n–3: omega–3 fatty acid series; T: tumor cells; MED: median; 95% CI: 95% confidence interval. Tumor cells: recurrent laryngeal papillomatosis (RLP). Control A: normal mucosa cells from larynx in RLP patient. Control B: normal mucosal cells from anterior tonsillar pillar of tonsillectomy patients.

Table 2a. Total phospholipids fatty acids in papilloma group compared with control group.

FA	Tumor vs. control			
	Med T	Med C	*p*	95% CI
16:0	23.7	25.0	0.82	-5.31; 4.76
18:0	23.7	34.5	0.02	-15.8; -0.88
18:1n–9	19.8	13.1	0.02	-0.28; 11.3
18:2n–6	25.9	14.2	0.01	0.87; 16.4

Abbreviations: C: control; FA: fatty acid; MED: median; MED: median; T: tumor cells; 95% CI: 95% confidence interval; 16:0: palmitic acid; 18:0: stearic acid; 18:1n–9: oleic acid; 18:2n–6: linoleic acid.

Table 2a. Total phospholipid fatty acid groups in papilloma group compared with control group.

FA	Tumor vs. control			
	Med T	Med C	*p*	95% CI
SFA	54.5	62.4	0.03	-14.6; 0.33
MUFA	21.4	0.0	0.02	-4.22; 17.8
PUFA	14.2	25.9	0.01	3.12; 16.4

Abbreviations: C: control; FA: fatty acid; MED: median; MUFA: monounsaturated fatty acid; n–6 PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; T: tumor cells; 95% CI: 95% confidence interval.

early phase HPV expression, whilst in the case of control group B (tonsillectomy patients), an early inflammatory reaction can not be ruled out. Nevertheless, our study did present results of statistical significance.

The papilloma profile showed significantly decreased n–6 PUFA ($p<0.04$) and n–3 PUFA ($p<0.02$) levels, and significantly increased heneicosanoic acid (HEA) (21:0) ($p<0.01$), eicosadienoic acid, (EDA) (20:2n–6) ($p<0.02$), eicosapentaenoic acid (EPA) (20:5n–3) ($p<0.02$) and docosahexaenoic acid (DHA) (22:6n–3)($p<0.02$) levels, when compared with control group B (Table 1a). No detectable trace of arachidonic acid (AA) (20:4n–6) occurred in the papilloma group, as well as both control groups (Table 1a).

Viral interference with the conversion of LA to AA and an oxidative burst: caused by harmful LA metabolite production, via 15-lipoxygenase activity; and reactive oxygen species and nitrogen oxide production under inflammatory conditions, characterized by enhanced cyclooxygenese-2 (COX-2) and PGE_2 expression, may decrease LA and absolutely exhaust AA sources.[10,11] Of importance is the significantly lower n–6 and n–3 PUFA levels in the papilloma cells and that the n-9 FA pathway with enhanced oleic acid (OA) (18:1n–9) and EDA (20:2n–6) are followed, when compared with control group B (Table1b). Of interest may be the higher relative % of PA (16:0) and SA (18:0) in the papilloma group, compared with control group B (Table 1a). This may reflect enhanced fatty acid synthase (FAS) and Δ^9d activities, respectively, to endogenously produced PA (associated with apoptotic resistance) and SA (for conversion to OA).[12,13] The conversion of OA (18:1n–9) to EDA (20:2n–6) by Δ^6d, but an accumulation of EDA (20:2n–6) with apparently no further conversion by Δ^5d, confirms that interference of these enzymes may occur at different steps during FA metabolic pathways. In the case of n–3 PUFAs, interference with the conversion of dietary intakes of αLA, EPA and DHA apparently occurred, while in both controls these insufficient dietary intakes were probably metabolized and exhausted.

RBC PL FA analysis, a true and reliable source of FA intake and metabolism, revealed an excessive LA intake of 23.6 g/d, compared with the 1.7g/d RDA.[14] Major FAs from this blood profile are the significantly enhanced SA ($p<0.01$), OA ($p<0.02$) and LA ($p<0.01$), as well as the significantly higher SFAs ($p<0.03$) in the papilloma group, compared with the control group, that regulates cellular immunity (Tables 2a and b).[15] SFA levels were also significantly higher than MUFAs and PUFAs within the papilloma and control groups, but only the PUFAs were significantly lower when the papilloma group was compared with the control group (data not shown). Enhanced dietary LA intake and saturation of papilloma cells are apparently responsible for a mitogenetic driven stimulus. Enhanced dietary SFA intakes are known to enhance COX-2 expression and a feedback mechanism exists whereby COX-2 activity stimulates the epidermal growth factor response that enhances cell proliferation via LA (and even AA), all conditions under which papilloma cells thrive.[16, 17] It is postulated that cumulative oxidative stress (with dietary LA and AA as the FA sources) and an oxidative burst is responsible for complete exhaustion of exogenous dietary AA in all the patients.

CONCLUSIONS

This study contributes to clinically relevant FA profiles for RLP, not previously reported in the literature. The role of LA in RLPs needs to be fully explored. It is suggested that LA, in part, may be converted to harmful lipoxygenase (LOX) products 9- and 13- hydroxyoctadecadienoic acid (9- and 13-HODEs), or, in part, to conjugated-linoleic acid-isomers (CLA). The AA exhaustion observed in our study may be attributed to oxidative stress caused by LA metabolites (9- and 13-HODEs), together with reactive oxygen species (ROS) and nitic oxygen species (NOS) produced under inflammatory conditions. These assumptions need to be confirmed or refuted. It is argued that enhanced SFA levels that regulate the Th1 cytokine subset[16] contribute to immunodeficiency that continues to trigger disease recurrence, discussed in part III of this study. It seems feasible that manipulation of membrane FA compositions may have beneficial use in the management of RLP patients.

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AUTHOR DISCLOSURES

Louise Louw, Riaz Seedat and André Claassen, no conflicts of interest.

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