Production of carcinogenic acetaldehyde by *Candida albicans* from patients with potentially malignant oral mucosal disorders

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OBJECTIVES: Production of carcinogenic acetaldehyde by *Candida* has been suggested to contribute to epithelial dysplasia and oral carcinogenesis. Oral lichen planus (OLP), oral lichenoid lesion (OLL) and oral leukoplakia (OL) are potentially carcinogenic oral diseases where colonisation by *Candida* is common, but acetaldehyde production by *Candida* has not been studied.

STUDY DESIGN: Acetaldehyde production in ethanol (11 mM), glucose (100 mM), ethanol–glucose (11 mM and 100 mM) or red wine (1200 mM ethanol) incubation by *Candida albicans* from patients with OLL (n = 6), OLP (n = 16), OL (n = 6) and controls (n = 6) was measured by gas chromatography. Participants completed a questionnaire regarding their smoking habits and alcohol consumption.

RESULTS: All *Candida albicans* isolates produced potentially carcinogenic levels of acetaldehyde (>100 μM) in all incubations containing ethanol. The control group isolates produced the highest acetaldehyde levels. Isolates from smokers produced more acetaldehyde in all incubations than those from non-smokers. The difference was significant in ethanol–glucose incubation. Isolates from patients who were both smokers and drinkers produced the highest amounts when incubated in ethanol, ethanol–glucose and wine.

CONCLUSIONS: *Candida albicans* isolated from potentially carcinogenic oral diseases can produce mutagenic amounts of acetaldehyde. Cigarette smoking and alcohol consumption may favour adaptational changes resulting in the upregulation of candidal acetaldehyde metabolism.

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Introduction

Oral lichen planus (OLP), oral lichenoid lesion (OLL) and oral leukoplakia (OL) are classified as potentially carcinogenic conditions or lesions (1). Various factors such as immune dysregulation, alcohol consumption and smoking have been identified in their etiopathogenesis (1–4). It has also been suggested that colonisation by *Candida albicans* may contribute to this as patients with these oral conditions and lesions often suffer from recurrent candidal infections (1, 5, 6). However, this may also be secondary to the underlying immunological dysregulation or to the use of immunosuppressive medications in the treatment (7). The prevalence of malignant transformation in OLP and OLL patients has been reported to be roughly 2% in 5-year follow-up, and this transformation does not appear to be restricted to the site of the lesion (1, 8, 9).

Alcohol consumption and tobacco smoking have an important role in oral carcinogenesis (10–13). It has recently been shown that 44% of upper aerodigestive tract cancers in men in Europe and 25% in women may be attributable to alcohol consumption (14). In the human body, ethanol becomes oxidised into acetaldehyde that is a highly reactive and toxic compound recently reclassified as a Group 1 carcinogen by the International Agency for Research on Cancer (12, 15). Acetaldehyde binds directly to DNA and may cause mutations at concentrations as low as 100 μM

(16–19). After moderate alcohol intake, marked levels of acetaldehyde can be detected in saliva for prolonged periods (20, 21). The oral microbiota has been shown to have a role in the salivary acetaldehyde production (20, 22, 23). *Candida* species have proved to have an extraordinary capacity to produce acetaldehyde from ethanol by oxidation as well as from glucose by fermentation (24, 25). High levels of acetaldehyde can also be detected in tobacco smoke (26).

Alcoholic beverages often contain acetaldehyde as a congener (18). During the process of alcohol fermentation, high amounts of acetaldehyde become produced from the various carbon sources (27). The concentration of acetaldehyde in wine is subject to the production process as well as the concentration of glucose and fructose and other constituents present in wine and produced during fermentation (18, 27, 28). Wine and other alcoholic beverages produced by fermentation and containing rather high concentrations of ethanol may contain more acetaldehyde than those beverages produced by distillation as highly volatile acetaldehyde escapes during the production process (18).

The role of candidal acetaldehyde production in potentially malignant oral disorders is unknown. Therefore, the aim of this study was firstly to examine the ability of *Candida albicans* isolated from various potentially malignant lesions to produce carcinogenic levels of acetaldehyde. Our hypothesis was that candidal acetaldehyde production may have a role in the malignant transformation of these lesions. Secondly, we wanted to analyse whether alcohol consumption and smoking habits of the patients have an impact on the candidal acetaldehyde metabolism. Regular alcohol consumption and smoking may lead to adaptation of *Candida* colonising the oral surfaces to repeated ethanol and acetaldehyde exposure.

Methods

Patients

A total of 28 patients referred to the Clinic of Oral Medicine Unit of the University of the Basque Country, Spain, during 2007–2010 with potentially malignant oral disorders and positive for *Candida albicans* by culture were included in the study (Table 1). Clinical assessment followed the standardised protocol by the Spanish Society of Oral Medicine (SEMO), and the clinicopathological diagnostic criteria of oral lichenoid disease (OLD; $n = 22$) (29) and oral leukoplakia (OL; $n = 6$) (3). The patients with OLD were further classified as oral lichenoid lesion (OLL; $n = 6$) or oral lichen planus (OLP; $n = 16$) following the criteria of van der Meij and van der Waal (30). Patients with recent or ongoing antifungal or steroid therapy were excluded from the study. Six patients with healthy oral mucosa but positive for *Candida albicans* by culture attending the Dental Clinic Service were recruited as controls. One isolate per study subject was included. All participants completed a questionnaire regarding their smoking habits and alcohol consumption and were stratified into smokers or non-smokers and drinkers or non-drinkers for the analyses. One cigarette or cigar was scored as one unit of tobacco, and one glass of wine (150 ml), one bottle of beer (330 ml) or one measure of spirit (35 ml) was scored as one unit of alcohol (31). The protocol was approved by the Ethics Committee (CEISH) of the University of the Basque Country/EHU, and each subject in the project signed a detailed informed consent form.

Candida albicans isolates

Candida albicans were isolated from patient samples using conventional culture methods and stored in the departmental culture collection at −80°C. Identification was based on colony morphology, growth on chromogenic agar (CHROMagar *Candida*, France), microscopy and biochemical reactions in API ID32C (Bio-Merieux, Marcy L'Etoile, France) assimilation tests. Identification was confirmed by molecular methods using PCR primers CR-r and CR-f as described earlier (32).

Acetaldehyde measurement

The method previously described by Nieminen et al. (24) was used for the measurement acetaldehyde production by *Candida*. *C. albicans* isolates were first incubated on Sabouraud agar (Oxoid, UK) at 37°C for 48 h prior to the experiment. Colonies of each isolate were suspended into phosphate buffered saline (PBS) and adjusted to a final concentration of 1×10^7 colony-forming units per millilitre (CFU/ml) by haemocytometer. A yeast suspension of 500 μl was transferred into vials containing 500 μl of ethanol (EtOH; final concentration of 11 mM) or glucose (final concentration of 100 mM) or ethanol and glucose (final concentration of 11 mM EtOH and 100 mM glucose) or red wine including 14.0% v/v ethanol (final concentration of 1200 mM EtOH,) (Marqués del Norte, La Rioja, Spain). The samples were sealed with silicon caps and incubated at 37°C for 30 min. The reaction was stopped by injecting 100 μl of perchloric acid (PCA; final concentration of 0.6 M). Three parallel samples were processed, and the experiment was repeated once. To measure the baseline and artefactual acetaldehyde, 100 μl of PCA was added immediately to control vials that contained yeast cells only, and the suspension was incubated for 30 min at 37°C. The formed ACH was measured by gas chromatography using the Varian CP-3800 equipped with a Zebron ZB-WaxPlus (Phenomenex Inc., Torrance, CA, USA) column and detection by flame ionisation (FID) and with automated CombiPal Headspace sampler (Agilent Technologies, Mississagua, ON, Canada) and subtracted from the initial values as reported earlier (24).

Statistical analyses

The results are expressed as means (±SEM). The data were analysed using SPSS statistical package 18.0 (SPSS Inc., Chicago, IL, USA). The generalised estimating equations model (GEE) was used for comparisons between species and experimental conditions, and for the analyses of correlations. Comparisons were made for groups with $n \geq 3$. Patients who were both drinkers and smokers were excluded from the comparisons owing to small numbers. The correlation was expressed with a 95% confidence interval. A P-value < 0.05 was considered statistically significant.

Table 1 Patient characteristics of *Candida albicans* isolates used in the study

Patient	*Age*	*Sex*	*Clinical presentation*	*Erosive*	*Oral manifestations*	*Other diagnostic findings*	*Smoking*	*Alcohol consumption*
OLL1	54	F	Reticular only	No	Asymmetrical	Histopathology	<10 U/day	No
OLL2	74	F	Reticular only	No	Asymmetrical		No	14 U/week
OLL3	65	M	Reticular + plaque	No	Asymmetrical		No	No
OLL4	81	F	Reticular + plaque	No	Asymmetrical	Histopathology	No	No
OLL5	49	M	Reticular + atrophic	No	Asymmetrical		No	49 U/week
OLL6	76	F	Reticular + atrophic	No	Asymmetrical	Histopathology	No	No
OLP1	40	F	Reticular only	No	Symmetrical		<10 U/day	No
OLP2	42	F	Reticular only	No	Symmetrical		No	No
OLP3	49	M	Reticular only	No	Symmetrical		No	No
OLP4	63	F	Reticular only	No	Symmetrical		No	No
OLP5	53	M	Reticular + atrophic	Yes	Symmetrical	Skin manifestations	No	7 U/week
OLP6	56	M	Reticular + plaque	No	Symmetrical		No	56 U/week
OLP7	76	F	Reticular + atrophic	Yes	Symmetrical	Skin manifestations	No	No
OLP8	76	F	Reticular + atrophic	Yes	Symmetrical		<10 U/day	No
OLP9	57	M	Reticular only	No	Symmetrical	Histopathology, skin manifestations	No	No
OLP10	58	M	Reticular only	No	Symmetrical	Histopathology	No	No
OLP11	59	F	Reticular only	No	Symmetrical	Histopathology	No	No
OLP12	36	M	Reticular + plaque	No	Symmetrical	Histopathology	>10 U/day	No
OLP13	55	F	Reticular + atrophic	Yes	Symmetrical	Histopathology	No	No
OLP14	57	F	Reticular + atrophic	Yes	Symmetrical	Histopathology	No	7 U/week
OLP15	83	F	Reticular + atrophic	Yes	Symmetrical	Histopathology	No	No
OLP16	74	F	Reticular + plaque + atrophic	Yes	Symmetrical	Histopathology	No	No
OL1	46	F	Homogeneous	No		Histopathology	>10 U/day	No
OL2	57	M	Homogeneous	No		Histopathology	>10 U/day	7 U/week
OL3	60	F	Homogeneous	No		Histopathology	>10 U/day	No
OL4	74	F	Homogeneous	No		Histopathology	>10 U/day	No
OL5	80	F	Homogeneous	No		Histopathology	>10 U/day	No
OL6	64	M	Non-homogeneous speckled (20)	No		Histopathology	>10 U/day	28 U/week
C1	21	F	–	–	–	–	No	2 U/week
C2	34	M	–	–	–	–	>10 U/day	No
C3	50	M	–	–	–	–	No	10 U/week
C4	54	F	–	–	–	–	No	2 U/week
C5	59	F	–	–	–	–	No	No
C6	59	F	–	–	–	–	>10 U/day	3 U/week

OLL, Oral lichenoid lesions (n = 6); OLP, Oral lichen planus (n = 16); OL, Oral leukoplakia (n = 6); C, Control patients (n = 6); U/day, units per day; U/week, units per week.

Results

All *Candida albicans* isolates produced high levels of acetaldehyde (>100 μM) in all incubations containing ethanol. The mean acetaldehyde production was 190.3 μM (±3.2 μM) in 11 mM ethanol, 229.7 μM (±3.5 μM) in ethanol–glucose (11 mM and 100 mM, respectively), and 111.7 μM (±3.4 μM) in wine incubation (1200 mM ethanol). However, in 100 mM glucose incubation, the mean acetaldehyde production was low (42.3 ± 1.3 μM). The isolates from smokers (n = 9) produced more acetaldehyde than the non-smoker (n = 22) isolates in all incubations (Table 2). This difference was statistically significant in ethanol–glucose incubation (P = 0.020). The alcohol drinker isolates (n = 8) produced more acetaldehyde than the non-drinker isolates (n = 23) in wine incubation, but the difference was not significant. The isolates from patients who were both smokers and drinkers (n = 3) produced the highest amounts of acetaldehyde of all subgroups in ethanol (mean 226.6 ± 7.8 μM), ethanol–glucose (260.5 ± 6.5 μM) and wine (125.8 ± 8.6 μM; P = 0.008, P < 0.001, P = 0.024, respectively).

Interestingly, isolates from the control group produced more acetaldehyde than the isolates from the other groups in ethanol (mean, 215.5 ± 9.6 μM) and ethanol–glucose (260.2 ± 8.4 μM) incubations (Fig. 1a,b). The production was significantly higher than that of the OLL isolates when exposed to ethanol (P = 0.011), ethanol and glucose (P < 0.001) or glucose alone (P = 0.032). However, the levels produced by the OLP and OL isolates were not significantly lower than those produced by the control isolates in ethanol–glucose, glucose or wine. In 100 mM glucose, the acetaldehyde production was low in all clinical groups (Fig. 1c). When exposed to wine, OLL isolates produced the highest levels of acetaldehyde (124.4 ± 7.7 μM) (P = ns, Fig. 1d). There was no significant difference in acetaldehyde production by isolates from patients with or without erosive OLD lesions in any of the incubations.

The proportion of smokers in the OLL and OLP groups was similar to that of the control group, whereas all patients in the OL group were smokers. The proportion of drinkers in the OLL and OLP groups was similar to that of the OL group, whereas the majority of patients in the control group reported regular alcohol consumption (Table 1).

When incubated in wine, the isolates from smokers with OLL, OLP or OL produced more acetaldehyde than the control isolates (172.3 ± 13.3 μM, 124.8 ± 9.1 μM and

Table 2 Mean acetaldehyde production (±SEM) by *Candida albicans* isolates from smokers and non-smokers (a) and regular alcohol consumers (drinkers) and non-drinkers (b)

(a)	*Non-smokers* (n = 22)	*Smokers* (n = 9)	P
Ethanol	184.3 ± 3.6	193.0 ± 7.3	0.601
Ethanol–glucose	215.9 ± 3.8	253.2 ± 7.4	0.020**
Glucose	40.3 ± 1.5	46.9 ± 2.8	0.320
Wine	104.4 ± 4.3	124.8 ± 5.8	0.107
(b)	Non-drinkers (n = 23)	Drinkers (n = 8)	P
Ethanol	187.2 ± 3.7	185.8 ± 7.4	0.922
Ethanol–glucose	232.0 ± 4.2	211.7 ± 7.1	0.226
Glucose	44.2 ± 1.6	36.5 ± 2.2	0.172
Wine	107.5 ± 4.1	118.5 ± 7.4	0.501

Significant differences marked (** $0.001 < P < 0.05$).

119.0 ± 8.5 μM, respectively). The isolates from smokers of the OLL group produced the highest levels of acetaldehyde (172.3 ± 13.3 μM). When incubated in ethanol–glucose, a similar trend in the acetaldehyde production could be seen: isolates from smoker patients with OLD produced significantly more acetaldehyde than non-smoker isolates ($P = 0.035$). When incubated in ethanol alone, the smoker isolates of the OLL, OLP and OL groups produced less acetaldehyde than control isolates (139.5 ± 9.1 μM, 221.2 ± 10.1 μM and 175.4 ± 11.1 μM, accordingly). In glucose incubation, the isolates from smokers with OL produced more acetaldehyde than the control isolates (59.0 ± 4.8 μM vs. 44.7 ± 5.4 μM).

Isolates from the OLP and control group patients who reported regular alcohol consumption produced higher amounts of acetaldehyde in wine incubation than those from non-drinkers within the same group (106.0 ± 11.7 μM vs. 101.3 ± 5.0 μM, 143.6 ± 13.0 μM vs. 65.8 ± 13.4 μM, accordingly). Surprisingly, in the OLL group, isolates from non-drinkers produced higher amounts of acetaldehyde than the isolates from drinkers when incubated in wine (136.9 ± 9.7 μM and 99.4 ± 9.4 μM, accordingly). In ethanol–glucose incubation, the OLP drinker isolates produced the highest levels of acetaldehyde in ethanol–glucose incubation (230.2 ± 8.2 μM). When incubated in ethanol alone, the OLD non-drinker isolates produced more acetaldehyde than the drinker isolates (187.6 ± 4.1 μM vs. 173.8 ± 5.0 μM, P = ns). When incubated in glucose alone, the drinker isolates of the control group were the highest producers of acetaldehyde (42.5 ± 4.5 μM).

Discussion

Candida albicans is part of oral microbiota in most healthy individuals (33, 34). Presence of yeasts in the oral cavity has also been linked to epithelial dysplasia and oral carcinogenesis (6, 35, 36). A number of *Candida* spp. is known to be able to produce carcinogenic levels of acetaldehyde in the presence of ethanol (24, 27). Levels above 100 μM have been reported to be able to form DNA adducts, DNA cross-links, sister chromatid changes and chromosomal aberrations (16). Acetaldehyde has also been shown to interfere with DNA repair mechanisms including inducing mutations in the p53 tumour suppressor gene (19). In the present study, *C. albicans* isolated from patients with potentially carcinogenic oral lesions or conditions produced mutagenic

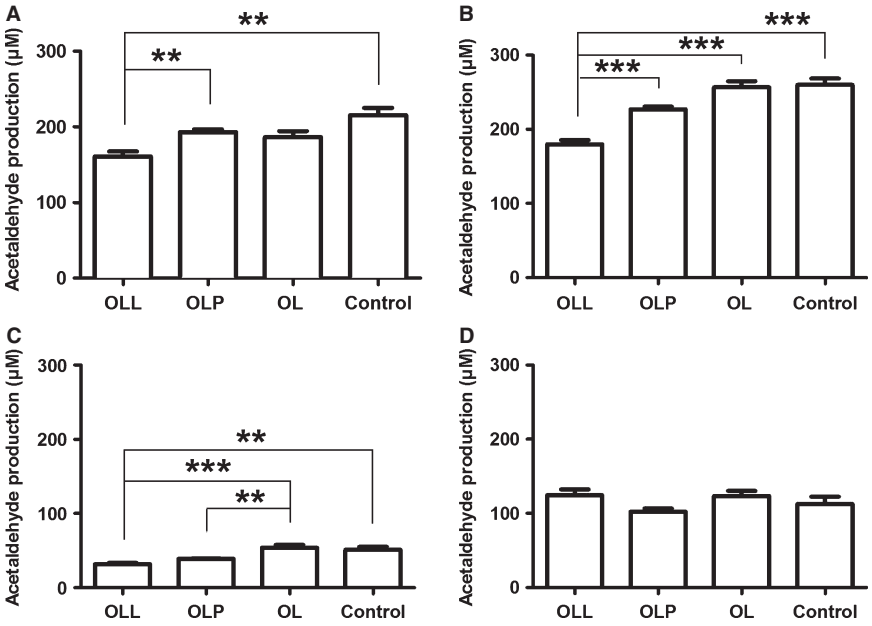

Figure 1 Mean acetaldehyde production by *Candida albicans* isolates from four different clinical groups oral lichenoid lesion ($n = 6$), oral lichen planus ($n = 12$), OL ($n = 6$) and controls ($n = 6$). The isolates were incubated in 11 mM ethanol (A), 11 mM ethanol and 100 mM glucose (B), 100 mM glucose (c), red wine (1200 mM ethanol final concentration) (d). The isolates were incubated at 37°C for 30 min. Error bars represent the standard error of mean (±SEM) within groups. Significant differences marked (***$P < 0.001$, **$0.001 < P < 0.05$).

levels of acetaldehyde in the presence of clinically relevant concentrations of ethanol, ethanol and glucose, and red wine (17). The concentrations of ethanol used in the present study can be found in blood and saliva after moderate alcohol consumption, and concentrations of glucose used in normal food and drinks (24). Although levels of acetaldehyde formed in glucose incubation were low, co-incubation with ethanol resulted in higher levels than in ethanol alone. Acetaldehyde production was slightly lower in wine than in the other ethanol-containing incubations. This is likely due to the high level of ethanol being toxic to *Candida* and the very low concentration of residual sugar as an alternative source of carbon in wine (37).

Interestingly, isolates of *C. albicans* from patients who reported to be smokers produced more acetaldehyde in all incubations than the isolates from non-smokers. The difference was significant in ethanol–glucose incubation. In wine, smoker isolates from all clinical groups produced more acetaldehyde than corresponding non-smoker isolates. Increased candidal acetaldehyde metabolism in smokers may in part explain the results of previous studies where high salivary acetaldehyde levels in heavy smokers were reported independently of their own ethanol metabolism (20, 38, 39). It is possible that *Candida* colonising the oral surfaces and repeatedly exposed to acetaldehyde in cigarette smoke becomes more tolerant to acetaldehyde and decreases its downstream metabolism via the citric acid cycle. It is also possible that acetaldehyde or the various other toxic agents in cigarette smoke provoke a general stress response in the candidal cells resulting in upregulation of anaerobic acetaldehyde-ethanol metabolism. These changes may be translational or the results of microevolution and selection. In our set of isolates, the likely explanations are genetic changes as the isolates were passaged multiple times in the absence of exposure to cigarette smoke before used in the experiments. Further studies are needed to address this point.

Isolates from patients who reported regular alcohol consumption produced higher acetaldehyde levels than isolates from non-drinkers, in ethanol, ethanol–glucose and glucose incubations. However, the differences between drinker and non-drinker isolates were statistically significant only in the control group. It is well known that most people under-report their alcohol consumption in non-anonymised questionnaires and self-reporting is highly unreliable (40, 41). Furthermore, smoking and alcohol consumption may have a cumulative effect on candidal acetaldehyde metabolism as isolates from patients who were both smokers and drinkers produced the highest amounts of acetaldehyde in ethanol, ethanol–glucose and wine. This is in accordance with previous work where smoking and alcohol consumption have been shown to have a synergistic effect on salivary acetaldehyde levels *in vivo* (39, 42). In most countries, high alcohol consumption and smoking have been more prevalent in men than in women, which is in line with the higher overall prevalence of aerodigestive tract cancers as well as with the higher prevalence of cancers potentially attributable to alcohol consumption in men (14). However, it is not known what proportion of those patients who develop oral cancer have had potentially malignant oral lesions and *Candida* colonisation. Nevertheless, as alcohol consumption increases, the role of candidal acetaldehyde production in the malignant transformation of OLD may increase.

Oral leukoplakia has been reported with a higher risk of malignant transformation than oral lichenoid disorder lesions (1). This has been linked to a higher exposure to other risk factors such as alcohol and tobacco in this patient group (1, 3). In our study, the isolates from the OL group produced significantly higher levels of acetaldehyde than isolates from oral lichenoid disorders when exposed to ethanol–glucose or glucose alone. In OL, high incidence of colonisation by *Candida,* high incidence of smoking and increased candidal acetaldehyde metabolism may all cumulatively contribute to the malignant transformation. The malignant potential of oral lichenoid disorders is controversial, and it has been suggested that this potential would be higher in OLL than in OLP lesions (9). In our study, although isolates from both lichenoid disorder groups produced carcinogenic levels of acetaldehyde, OLP isolates produced higher levels than isolates of the OLL group.

In conclusion, our *in vitro* study shows that *Candida albicans* isolated from potentially malignant oral disorders are able to produce mutagenic amounts of carcinogenic acetaldehyde when exposed to substrates such as wine and ethanol. Acetaldehyde produced locally by *Candida* may contribute to the pathogenesis of these oral disorders and to their malignant transformation. Oral cancer is a multifactorial disease and, in general, accumulation of risk factors is required for the malignant transformation. Chronic candidal infection resulting in local production of a carcinogen can alone explain some cases of oral cancer in patients with no other risk factors, and it certainly can contribute to the higher incidence of cancer in patients with potentially malignant oral mucosal diseases. Cigarette smoking and alcohol consumption may favour adaptational changes resulting in upregulation of candidal acetaldehyde metabolism. However, a spectrum of other factors may affect the metabolic activity of *Candida* on oral surfaces *in vivo,* whereby further studies in a more complex setting resembling *in vivo* conditions are warranted to elucidate the effects of candidal acetaldehyde production on mucosal cells.

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Conflict of interest

The authors report no conflicts of interest.