RESEARCH ARTICLE

Campylobacter Concisus and Its Effect on the Expression of CDX1 and COX2

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Abstract

Background: Barrett's oesophagus (BO) is a pre-malignant condition in which normal squamous epithelium of the lower oesophagus and gastresophageal junction is replaced by columnar cells and progress to oesophageal adenocarcinoma. The increase burden of oesophagus cancer morbidity and mortality worldwide make study of factors involved in the pathogenesis of BO essential. However, most of studies that examine the environmental risk factors associated with increased incidence and prevalence of BO have largely ignored the potential role of bacteria in disease aetiology. **Aims:** This study examined the role of *Campylobacter concisus* isolated from Barrett's and adenocarcinoma patient samples as one of possible environmental factors in the progression of Barrett's oesophagus to oesophagus adenocarcinoma. **Methods:** We focused on the effect of *C. concisus* on the expression caudal type homeobox 1 gene (CDX1) and cyclooxygenase-2 (COX-2) in three BO cell lines using quantitative real-time PCR. In addition, the attachment and invasion characteristics of *C. concisus* were also tested. **Results:** Results showed that *C. concisus* had a strong attachment to the cell lines and induce the expression of CDX1 in Barrett's cell lines in a time-dependent manner. **Conclusion:** Findings indicate that *C. concisus* could be as a new challenge in the progression of BO to adenocarcinoma.

Keywords: *Campylobacter concisus*- Barrett's oesophagus- COX2- CDX1

Introduction

Barrett's oesophagus (BO) is a condition in which stratified squamous epithelium lining the oesophagus is replaced by columnar shape epithelium and can develop into the oesophageal adenocarcinoma (OA). Although gastro-oesophageal reflux disease (GORD) is the main cause of BO, the mechanisms involved in the progression of BO to OA are unclear. The prevalence of BO is about 10-14% of individuals with GORD and in OA is 0.2% to 2.1% (Jonge et al., 2014). In the past three decades, data show that the prevalence of OA has increased in the UK and the Western Hemisphere (Gibson et al., 2013). It has been demonstrated that the progression of BO to OA involved multi-step alterations of gene expression, epigenetic, and/or microenvironmental factors (Leung et al., 2014).

Objective

Metaplastic changes of BO epithelium create a new microenvironment, which is colonized by a variety of bacterial species. These organisms compete with each other and try to evade/manipulate host immune system in order to circumscribe host responses. Bacteria metabolite

and toxin induce chronic inflammation, which drives or exacerbate neoplastic changes. Evidence shows that more than 15% of carcinogenesis can be attributed to the bacterial infection for instance Helicobacter pylori role in gastric cancer and mucosal-associated lymphoid tissue lymphoma, Streptococcus bovis in colon cancer, and Chlamydia pneumonia in lung cancer. This is possible by host cell invasion, alternation of cell cycle, DNA damage, subversion of cell division and apoptosis, and releasing different cytokines (Leung et al., 2014).

Studies on mucosal biopsies of BO and OA patients have demonstrated that bacterial community shift from Gram positive to Gram negative bacteria including the predominance of Campylobacter population with a high preponderance of *C. concisus* (Rosenvinge et al., 2013). *C. concisus* pathogenicity has been studied in periodontal disease, children's diarrhoea and inflammatory bowel disease (Macfarlane et al., 2009; Kaakoush et al., 2011). The pathogenic potential of *C. concisus* and its association with intestinal diseases make them a good candidate for potential pathogen in BO. This association might involve one of those mechanisms presented by Yang (2012) such as modulating CDX1 and COX2 signalling pathway through bacterial lipopolysaccharide. Consequently,

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evaluating effect of *C. concisus* on the expression of COX2, and CDX1 might highlight new aspect of microbial involvement in BO progression. This study aimed to explore the effect of *C. concisus* in the modulation of these biomarkers on a cell culture model with three oesophageal cell lines.

Materials and Methods

Bacterial strains

The *C. concisus* strain used in this study were isolated from mucosal biopsies of OA patients and healthy volunteers as described by Mozaffari namin (2015a). Briefly, *C. concisus* was grown and sub-cultured every three days on Wilkins–Chalgren (WC) agar (Oxoid, Basingstoke, Hamps, UK), and incubated at 37°C under an atmosphere of 10% H_2, 10% CO_2 and 80% N_2, in an anaerobic cabinet (Don Whitley, Shipley, UK).

Epithelial cell culture and co-culture assays

Three cell lines were used in this study; the Barrett's-associated adenocarcinoma cell line FLO-1, CP-A (non-dysplastic metaplasia), and CP-D (high-grade dysplastic metaplasia), described previously Mozaffari Namin et al., (2015a). In brief, FLO-1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK), and CP-A and CP-D cell lines were maintained in keratinocyte serum-free medium (KSFM, Invitrogen, UK). For co-culture assays, cells were then seeded at 5×10^5 cells ml^{-1} into 6-well micro plates (Corning, UK), in 3 ml of complete medium. Cells were allowed to form semi-confluent monolayers for adherence and invasion assays.

In vitro adhesion assays

To determine the pathogen's adhesive abilities, FLO-1 cells were grown on 15×15 mm sterile glass coverslips in 6-well tissue culture plates, and incubated as described previously. Coverslips were then washed three times with PBS and incubated in new antibiotic-free media containing 3×10^7 ml^{-1} of *C. concisus*. Plates were incubated at 37°C for 15 min, 1 and 3 hours. At each time point, coverslips were washed gently three times by dipping in warm PBS to remove non-adherent bacteria. Adherent bacteria were stained by two different methods; safranin and propidium iodide. In safranin staining, the cells were fixed in 95% methanol for 5 min, dried, and performed Gram stain only with safranin, and live/dead staining with propidium iodide (1.5 μlml^{-1}) as described previously by Macfarlane et al., (2004). Each assay was performed in duplicate and repeated three times. Number of adherent bacteria was counted in 20 randomly selected microscopic areas then viewed and counted by fluorescent microscopy.

In vitro invasion assays

Approximately 3×10^7 ml^{-1} bacteria were added to confluent FLO-1 cell monolayers in 6-well micro plates and incubated as descried above, for 0, 1, 3, 5, 7 hours. Subsequently, extracellular bacteria were killed by addition of 200 µg ml^{-1} gentamicin as a routine lab method to eradicate extracellular bacteria and its

concentration was useful for incubation time. Cells were washed three times with PBS, and intracellular bacteria were enumerated after lysis of the cells using 1% Triton X-100 in PBS, and plate counting.

mRNA isolation and real-time quantitative PCR

Expression profiles for selected biomarker genes by FLO-1 and BO cell lines after stimulation by *C. concisus* were determined relative to the GAPDH housekeeping gene by extraction of mRNA, cDNA synthesis, and quantification by real-time PCR (qPCR), as described below.

FLO-1, CP-A and CP-D cells challenged with *C. concisus* were harvested and total RNAs were isolated using RNA easy kit (Qiagen, UK), including 15 min on-column DNase step and reverse transcribed using the Quick reverse transcription system (Promega) with specific primer pairs (Table 1) as per the manufacturer's instructions. Synthesised cDNAs were aliquoted (2 µl) and stored at -80°C prior analysis as described by Bahrami et al., (2014). Briefly qPCR was carried out done using an iCycler detection system and iQ SYBR Green Supermix. Test samples were added in triplicate at 2 µl per well in a 20 µl total reaction volume with appropriate annealing temperature (Table 1). Target gene copy numbers were then extrapolated from appropriate standard curves and normalised against the house keeping gene (GAPDH) using ΔCT method. Results are expressed as averages of three separate experiments.

Statistical analysis

Data are reported as means ± standard errors of mean (SEM). Repeated-measurement two-way ANOVA analysis followed by Bonferroni post-hoc test was used for statistical analysis between different groups. P values of <0.05 was considered as statistically significant. Data analysis was undertaken using the GraphPad Prism, version 4 (GraphPad Software Inc, San Diego, CA).

Results

Bacterial adhesion

Bacterial adhesion assay of BO cells was investigated as a prerequisite for cell invasion. This was assessed at different time points by confocal microscopy through the live/dead staining and safranin staining. Adhesion observations displayed that *C. concisus* aggregated and formed microcolonies to increase its adhesion abilities, but it was unable to invade the BO cell lines (Figure 1).

Effects of C. concisus on cellular biomarker expression

Molecular analyses showed that significant difference was observed on the expression of CDX1 after 3-hour in FLO-1 cell line (p <0.001) (Table 2), while it was not detectable in both CP-A and CP-D cell lines (Table 3, 4). Results of co-culture in the COX2 expression showed that it has not been detected in FLO-1 cell line (p <0.001) (Table 2) and its expression was no more than control in both cell lines, CP-A and CP-D (p<0.001) (Table 3, 4).

Table 1. Primer Sequences Used in the Real-time Quantitative PCR and Their Characteristics

Primer set		Primer sequence (5¢-3¢)	TM (°C)	Product size (bp)	Reference
Campylobacter concisus		F: CAGTATCGGCAATTCGCT R: GACAGTATCAAGGATTTACG	60	306	(Enberg J and Bang, 2005)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F:GGAAGGTGAAGGTCGGAGTC R:TCAGCCTTGACGGTGCCATG	56	-13	(Furrie et al., 2007)
COX2	Cyclooxygenase-2	F:GTGTTGCGGAGAAAGGAGTCAT R:ACTTTCAGCATTTTGGCATCTTG	64	-14	(Maaser et al., 2008)
CDX1	Caudal related homeobox 1	F:AGCGCAAAGTGAACAAGAAGAAACAG R:GGGGCTATGGCAGAAACTCCTCT	60	-15	(Mizoshita et al., 2013)

TM, annealing temperature; bp, base pairs

Table 2. Fold Change mRNA Expression for Biomarkers in FLO-1 Cell after Co-culture with *C. concisus*

	CDX1		COX2	
Time	T	C	T	C
1h	932 ±1.65	7181 ± ND	ND	41341 ± 3
3h	10122 ±2.65	8365 ± ND	ND	38238 ± 2.16
5h	970 ±1.45	7033 ± ND	ND	44667 ± 3.45
7h	861 ±1.72	8511 ± ND	ND	35342 ± 3.2
P value	**		**	

FLO-1 cells were stimulated with C. concisus for 1–7 h, samples mRNA was isolated, cDNA were prepared and real-time PCR analysis was carried out using specific primers for CDX1, COX2 and GAPD. Data are presented as mean ± SEM of triplicate experiments. Quantitative PCR analysis of stimulated cells compared with non-stimulated controls, analysed by two-way ANOVA followed by Bonferroni post-hoc test analysis. T, test; C, control (cell without bacteria); ND, not detected. **, P < 0.001 significantly different from control values.

Table 4. mRNA-fold Differences Expression for Biomarkers in CP-D Cell Lines after Co-culture with *C. concisus*

	CDX1		COX2	
Time	T	C	T	C
1 h	ND	9747 ± 2.6	43 ± 1.89	4716 ± 1.3
3 h	ND	12000 ± 3.1	79 ± 1.2	6000 ± 1.16
5 h	ND	12000 ± 3.1	227 ± 1.54	6930 ± 1.45
7 h	ND	10055 ± 3.81	400 ± 2.3	6324 ± 1.23
P value	**		**	

CP-D cells were challenged with organism and then after extracting mRNA and preparing cDNA qPCR was conducted. Results are shown as means ± SEM from three independent experiments compared with non-stimulated controls, analysed by two-way ANOVA followed by Bonferroni post-hoc test analysis. T, test; C, control (cell without bacteria); ND, not detected. **, P < 0.001 significantly different from control values.

Discussion

Discussion and Conclusion

Most of researches on the etiology of BO have focused on various risk factors taking into account both environmental and genetic factors (Lim and Fitzgerald, 2013). However, these studies did not adequately address the role of environmental factor such as bacteria. We propose bacteria as a key risk factor for BO, focusing on the role of *C. concisus*. Our results showed that *C. concisus* could modulate expression of

Table 3. mRNA-fold Differences Expression for Biomarkers in CP-A Cell Lines after Co-culture with *C. concisus*

	CDX1		COX2	
Time	T	C	T	C
1h	ND	1327± 1.6	19± 1.2	184 ± 1.3
3h	ND	1575 ± 2	35± 1	170 ± 1.16
5h	ND	1619 ± 1.85	65± 1.1	220 ± 1.45
7h	ND	1420 ± 1.35	100± 2	231 ± 1.23
P value	**		**	

CP-A cells were challenged with organism and then after extracting mRNA and preparing cDNA qPCR was conducted. Results are shown as means ± SEM from three independent experiments compared with non-stimulated controls, analysed by two-way ANOVA followed by Bonferroni post-hoc test analysis. T, test; C, control (cell without bacteria); ND, not detected. **, P < 0.001 significantly different from control values.
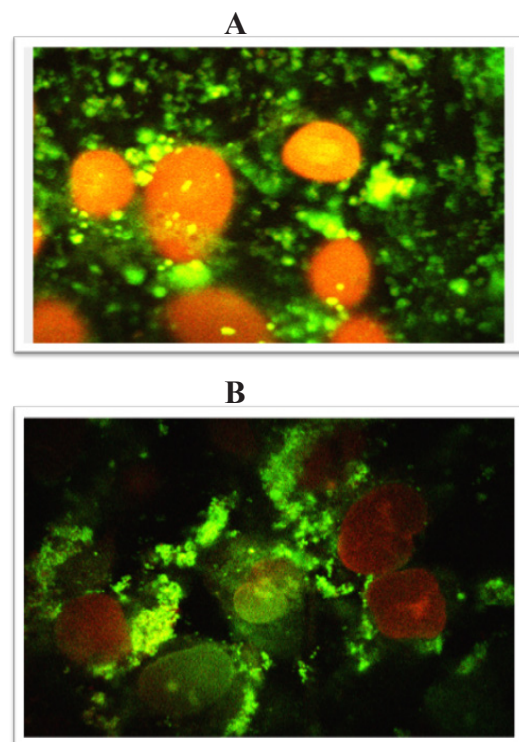
A

B

Figure 1. Fluorescence Microscopy Images of CP-D Cell Line co-cultured with *C. concisus*. (A) Live and dead staining with propidium iodide after 15 minute co-culture which shows bacteria are motile around the cells. Cells are shown in red colour and microbes are in yellow and green. (B) Staining with propidium iodide after 90 min, arrow shows bacteria have started to aggregate and surround the cells. Original magnification, ×100.

CDX1 in FLO-1. Current studies have shown organism could induce Toll-like receptors (TLRs) and expression of COX2 in HT-29 cell line (Ismail et al., 2013). Data presented by Blackett et al., (2013) revealed initiation expression of different cytokines (TNFα, IL18), and p53 (Mozaffari namin et al., 2015b) and also their expression in a primary study on BO and OA patient samples were all the effect of *C. concisus*. In addition, different studies have shown increased expression of CDX1 in BO has induced cell differentiation (Tamagawa et al., 2012) and alternation in the p53 expression resulting in increasing BO transition (Gajjar et al., 2012). These increase possibility of bacterial impacts on the expression of biomarkers involved in the progression of BO to OA or Barrett's pathogenesis.

Campylobacters express various virulence factors that enable them to adhere and invade host defence mechanisms. Different potent cytotoxic proteins and enzymes, such as haemolysin and cytolethal distending toxin (CDT) facilitate campylobacter virulence (Kaakoush et al., 2010). *Campylobacter concisus* ATCC13826 synthesizes two types of toxins, ZOT (zonula occludens toxin) and CDT, secretes 86 proteins, and has 25 genes associated with virulence or colonization activities (Kalischuk et al., 2011; Kaakoush and Mitchell, 2012).

Adhesion study presented that there were high accumulations of bacterium around BO cells (FLO-1, CP-D). This was similar to that reported by Man (2010) in which intestinal Caco2 and HT29 cell lines invaded by this organism. He documented that campylobacter species in particular *C. concisus* could modulate barrier permeability by attachment. Attachment is the first mechanism of bacterial virulence factor to facilitate injection of invasion antigen and toxins (CDT and ZOT), increase attachment of specific sticky end of bacterium, induce expression of pro-inflammatory cytokines, activate entry into host cell, control cytoskeletal or junctional function, and change in tissue stem cell homeostasis (Man et al., 2010; Vogelmann et al., 2007).

CDXs gene was the first selected biomarker in this study. Our finding revealed that *C. concisus* induces high level of CDX1 in FLO-1. There are number of activated genes in BO compared with normal oesophageal that might play different role but among them CDXs genes (CDX1 and CDX2), CDX1 plays a central role in the development of metaplasia and induction of cellular differentiation in oesophageal epithelial cells, while CDX2 is regulated by various environmental factors (Makita et al., 2013). This process has been detected in BO (Tamagawa et al., 2013), in gastric cancer related to H. pylori and in intestinal metaplasia and dysplasia with frequent expression of CDX1 and CDX2 (Kang and Lee, 2011). In addition, microarray analysis of normal oesophagus, Barrett's tissues, and small intestinal biopsies have shown significant expression of CDX1 and c-myc. It has been reported that overexpression of CDXs genes in the glandular epithelium of the mice stomach lead to intestinal metaplasia. These show Cdx transcription factors can change glandular cell fates which reveal overexpression of Cdx1 duo to the effect of *C. concisus* might play a role in BO (Stairs et al., 2008).

The next step of our study was to observe the role of

COX2 gene. In vitro studies have shown overexpression of COX2 in angiogenesis, inhibition of immune surveillance, reduction of apoptosis, cell adhesion, decreasing E-Cadherin expression, increased cell proliferation, and increasing invasive or metastatic potential and their role in oesophageal cancer cell lines via the effect of bile acid by inhibition of COX2 expression resulted in apoptotic cell death, proliferation activity, and prostaglandin E2 synthesis (Hashimoto et al., 2012). Beside, data display gradual enhancement in the expression of COX2 throughout the sequence of BO to malignancy, reduction of CDX2 and increase in CDC2 (cell division cycle 2) (Villanacci et al., 2006). Evidence indicate expression of COX2, CDX2, and MUC2 on direct samples of BO which show high level of their expression in the intestinal metaplastic epithelium than in distant and non-goblet columnar epithelium (Gajjar et al., 2012). However, our finding showed that although *C. concisus* suppressed expression of COX2 in BO cell lines, the COX2 expression had slight increase between metaplastic (CP-A) and dysplastic (CP-D) cells. Investigations have shown that any alternations in the p53, CDX1, and COX2 genes would results in various abnormalities and genetic damage. This variation in BO results in 16-fold higher risk of OA than those without any changes in these markers. Even, molecular epidemiology has revealed that the prevalence of positive p53 immunoreactivity in different stages of Barrett's progression linked to significant rates of p53 overexpression. This indicates that p53 in concert with other tumour progression markers is switched on in early stages of BO due to environmental factors and leads to abnormalities in the cell cycle (Turkmen et al., 2012). Chronic inflammation can influence unrestrained proliferation which leads to production of unwanted proteins as a result of CDXs genes or p53 gene (Hritz et al., 2009). Since apoptosis is usually programmed by p53 to discount death or abnormal cells in cell cycle by binding to TNF receptors and Fas, any disruption in this pattern will results in unwanted cells. Similarly, cell proliferation and p53 overexpression in OA patient samples compared to healthy group have been reported to account for the progression of BO to OA (Fichter et al., 2011). Thus, association between the effect of COX2 and p53 and CDXs in the progression of BO could initiate risk of OA (Akutko and Matusiewicz, 2017). Providing that *C. concisus* can induce the operation of signalling pathway links to the activation of p53, it will create genetic instability leading to the establishment of cellular abnormalities and activating expression of CDX1 and COX2 (Mozaffari Namin et al., 2015b).

Each of the studied molecular markers expresses at different stages of BO transformation but there are close relationship between their activation and the induction of multiple cellular pathways in OA. We therefore considered gene expression profiles of key signalling factors in infection with *C. concisus* and also in current studies (Akutko and Matusiewicz, 2017; Di Pilato et al., 2016) there have been considered its role as a new agent in gastrointestinal diseases. Findings stated that gastric cancer is caused by long-term co-existence of *H. pylori*

with host cells. This extended process associates with a robust modulation of different signalling keys (such as p53 mutation, TNFα and IL18 modulation, and APC) and induction a multiple mechanisms from mucosal responses (Toll-like receptors) to the immune responses. These results point to might bacterial involvement/roles in BO transition to focus in more investigation.

In conclusion, despite unclear cellular and molecular mechanism of BO transition to AE, we postulate that BO creates new microenvironment that facilitates BO biofilm formation specifically *C. concisus* which in turn induce an inflammatory microenvironment favourable for induction or exacerbation of BO progression to OA. However, it still need more investigation to been answered.

Acknowledgments

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