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The Effect of *Campylobacter concisus* on Expression of IL-18, TNF-α and p53 in Barrett's Cell Lines

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Abstract

Background: Barrett's oesophagus is a pre-malignant condition at gastroesophageal junction in which normal squamous epithelium is replaced by columnar shape epithelium, which predisposes oesophageal adenocarcinoma. It is known that Barrett's oesophagus evolves as a consequence of chronic gastro-oesophageal reflux disease. Although progression of Barrett's oesophagus to adenocarcinoma is still unclear, increasing incidence of oesophageal cancer and mortality worldwide make its study necessary. Several investigations have been made on the aetiology of oesophageal cancer. Most of them assessed genetical or environmental factors. However, potential role of bacteria in the development of oesophageal adenocarcinoma as a new environmental factor has not been addressed. Previous study on Barrett's disease detected presence of *Campylobacter concisus* as a new emerging pathogen on Barrett's and oesophageal cancer samples compared with healthy individuals. This indicates that this organism might involve in the progression of Barrett's to oesophageal adenocarcinoma.
Objectives: This study aimed to determine the effects of *C. concisus* on expression of three biomarkers including interleukin-18 (IL-18), tumour necrosis factor-α (TNF-α) and tumour suppressor gene (p53) in three Barrett's cell lines.
Materials and Methods: Quantitative real-time PCR assays were developed to measure expression of pro-inflammatory mediators (IL-18 and TNF-α) and gene expression of p53 in Barrett's cell lines in co-culture with *C. concisus*.
Results: The mentioned organism was able to modulate considerably expression of p53, TNF-α and IL-18 in a time-dependent manner.
Conclusions: The results showed that microorganism influences expression of carcinogenesis biomarker and cytokines in cell line models and possibility promotes oesophageal adenocarcinoma.

Keywords: Barrett Oesophagus, Interleukin-18, Tumour Necrosis Factor-alpha (TNF-α), Genes, p53, *Campylobacter concisus*

1. Background

Barrett's oesophagus (BO) is a premalignant disorder at the end of oesophagus due to gastroesophageal reflux disease (GORD). It is known that BO is a progenitor of Oesophageal Adenocarcinoma (OA), which is a major cause of cancer death worldwide. Prevalence of BO is 0.2 - 2% annually in Western, Europe and the UK with 3-fold increase on the rate of OA over the last three decades. There have been various studies along with effective factors involved in the progression of BO to adenocarcinoma. Most of them have been focused on two major areas, genetical and/or environmental factors (1, 2). Recent evidences have revealed that alternation on oesophageal epithelium could result in colonising various pathogenic and non-pathogenic bacteria duo to new microenvironment. Study on BO biofilm showed that there are high level of atypical nitrate-reducing *Campylobacters* (especially *Campylobacter concisus*) in BO, compared with non-Barrett's samples (3, 4). *Campylobacter concisus* is a Gram-negative oral bacterium, which is supposed to be associated with inflammatory bowel disease, Crohn's disease and ulcerative colitis (5-8).

Campylobacter genus comprises 16 species and 6 subspecies, including important pathogens of humans and animals. The most significant species are *C. jejuni*, *C. coli*, *C. fetus,* and *C. rectus*, which cause a variety of infections such as gastroenteritis campylobacteriosis and periodontitis (9). Over the last decade, *C. gracilis*, *C. upsaliensis* and *C. lari*, especially *C. concisus*, have been linked to intestinal disease (8). Campylobacters express various virulence factors enabling them to adhere, invade and contribute to the evasion of host defence mechanisms. Different potent cytotoxic proteins and enzymes facilitate campylobacter virulence, such as haemolysin and cytolethal distending toxin (CDT) (10). Findings showed that *C. concisus* secretes 86 proteins, consists of 25 genes associated with

virulence or colonisation activities (6, 10, 11) and contains genotype and phenotype diversity (8). It can convert nitrate to nitrite, which leads to production of nitric oxide (carcinogenic agent). Clemons et al. (12) confirmed this characteristic of organism that how produced nitric oxide can affect the BO progression.

These all highlight ability of organism in adhesion and invasion of host cells. Evidence shows that microbe can invade intestinal epithelial cells, increase intestinal permeability, induce epithelial apoptosis in Caco2 cell line (13, 14) and interestingly induce inflammatory cytokines such as IL-6, IL-8, IL-10 in HT-29 (15), and IL-18 and TNF-α in BO cell lines (4). Considering these, data highlighted this hypothesis that *C. concisus* might play potential role in the aetiology of OA, an in vitro model of study was conducted to detect impact of organism on BO cell lines.

2. Objectives

This study aimed to investigate possible role of *C. concisus* in the expression of IL-18, TNF-α and p53 in BO cell lines as part of inflammatory key factors and intracellular signalling.

3. Materials and Methods

3.1. Bacterial Strains

Campylobacter concisus and *Streptococcus salivarius* strains used in this study had been isolated previously by Blackett et al. (4) from mucosal biopsies obtained from OA patients and healthy volunteers. Bacterial identities were verified by 16S rRNA gene sequencing at Ninewells hospital, Dundee, UK and Blast search identified the most closely related strains to be *C. concisus* ATCC13826 and *S. salivarius* with accession number NC015760.1. Main organism was grown and sub-cultured on Wilkins-Chalgren agar (WC; Oxoid) supplemented with formate (0.6 gl^{-1}), fumarate (0.6 gl^{-1}) (Sigma, Poole, Dorset, United Kingdom) in an anaerobic cabinet with 10% H_2, 10% CO_2 and 80% N_2 at 37°C (Don Whitley, United Kingdom). Before each test, organism was inoculated into WC broth in universal and incubated anaerobically at 37°C. Bacteria were then centrifuged (2500 × g, 25 minutes), washed once by PBS, counted in haemocytometer and resuspended to concentration of $3 \times 107\ ml^{-1}$. *S. salivarius* was grown aerobically in WC broth at 37°C for use as a negative control, as part of the normal human oesophageal microbiota (16).

3.2. Cell Culture

The Barrett's associated adenocarcinoma cell line FLO-1 (immortalised Barrett's oesophageal epithelial cells) and two Barrett's cell lines (CPA (non-dysplastic metaplasia) and CPD (high-grade dysplastic metaplasia)) were used to challenge with organism as described by Mozaffari Namin et al. (16). Briefly, FLO-1 was maintained in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, Gillingham, UK) with 10% foetal calf serum (FCS) (Gibco-BRL life technologies, UK) and 1% penicillin/ treptomycin (Gibco-BRL, UK). Barrett's cell lines in keratinocyte serum-free medium (KSFM) supplemented with 30 mgl-1 bovine pituitary extract (BPE), 0.2 μgl-1 recombinant epidermal growth factor (EPG) and 1% penicillin/streptomycin. Grown confluent cells were infected with pathogen and incubated at 37°C in 5% CO_2-95% O_2 in humidified atmosphere for 1, 5 and 7 hours. At the end of each time point, supernatant was aspirated off, cells were harvested, centrifuged (1000 × g for 5 minutes) and cells pellets were kept at −80°C for the rest of experiments. All cocultures were performed in triplicate and culture medium without bacteria was negative control.

3.3. RNA Extraction, cDNA, qPCR

Gene expression profile of biomarkers were determined by relative target gene quantities from threshold cycles (C_t) normalised against the housekeeping gene GAPDH in FLO-1and BO cell lines through the mRNA extraction using the RNA easy kit (Qiagen, Crawley, UK), cDNA synthesis using the Quick reverse transcription system (Promega) and quantification by real-time PCR (qPCR) with primer sets after stimulating by *C. concisus* as described previously by Mozaffari Namin et al. (16). Briefly, products of correct size and sequence were purified using the Qiaquick PCR purification kit and ligated into a vector using the pGEM-T easy vector system I (Promega). Plasmid DNA from candidate clones was isolated and purified using the Wizard plus SV miniprep system (Promega) and the sequence identity verified. Samples were diluted to 10^{10} molecules μl^{-1}, aliquoted and stored at −80°C. qPCR was performed using an iCycler real-time PCR detection system (BioRad laboratories, Hercules, CA, USA) in the presence of iQ SYBR Green Supermix (BioRad). The standard amplification protocol consisted of an initial denaturation step (95°C, 3 minutes), followed by 38 cycles of 95°C for 30 seconds, 30 seconds at appropriate annealing temperature (Table 1) and one final denaturation cycle (95°C, 30 seconds).

3.4. Statistical Analysis

GraphPad Prism, version 4 (GraphPad software Inc. San Diego, CA) was used for data analysis. Repeated-measurement two-way ANOVA analysis with Bonferroni post-hoc test was used for comparison of different groups. P Values < 0.05 were considered as statistically significant.

4. Results

4.1. Effects of Campylobacter concisus on Cellular Biomarker Expression

Molecular analyses showed that *C. concisus* has had remarkable effects on the expression of three cellular biomarkers. Significant differences were observed on the expression of p53 ($P < 0.001$), TNF-α ($P < 0.05$) and IL-18 ($P < 0.001$) in FLO-1 cell line compared to the control (Table

2). The highest difference on the expression of p53 was detected on CP-A cells after one hour ($P < 0.01$). TNF-α and IL-18 had not been expressed considerably in this cell line, although there was a slight increase during time points (Table 3). Significant folds gene expression of IL-18 was defined on CP-D cells where there was a gradual increase between 1 and 7 hours ($P < 0.001$). However, the impact of bacterium was not as much as control on the expression of TNF-α and p53 (Table 4).

4.2. Effects of Streptococcus salivarius on Cellular Biomarkers

Streptococcus salivarius was selected as a commensal bacterium to test its effects on the expression of selected biomarkers in contrast to *C. concisus*. Results showed no significant effects on the expression of biomarkers except a slight increase on p53 expression, which might be test error (Table 5).

Table 1. Quantitative PCR Primer Sequences and Characteristics[a]

Primer Set	Primer Sequence (5' - 3')	Target Group	Tm, °C	Product Size, bp	Reference
Camp		*Campylobacter concisus*	60	306	(17)
F	CAGTATCGGCAATTCGCT				
R	GACAGTATCAAGGATTTACG				
IL-18		interleukin 18	65	105	this study
F	GACGCATGCCCTCAATCC				
R	CTAGAGCGCAATGGTGCAATC				
TNF-α		tumour necrosis factor-α	56	123	(18)
F	TCTCGAACCCCGAGTGACAA				
R	TATCTCTCAGCTCCACGCCA				
GAPDH		glyceraldehyde-3-phosphate dehydrogenase	56	183	(18)
F	GGAAGGTGAAGGTCGGAGTC				
R	TCAGCCTTGACGGTGCCATG				
p53		tumour suppressor gene	61	120	(19)
F	CAGCCAAGTCTGTGACTTGCA				
R	GTGTGGAATCAACCCACAGCT				

[a]Abbreviations: Tm, annealing temperature; bp, base pairs.

Table 2. mRNA-Fold Gene Expression Differences on the Expression of Biomarkers in FLO-1 Cells Co-Cultured With *C. concisus*[a,b]

Biomarkers	Time, h				P Value
	1	3	5	7	
p53					< 0.001
T	677 ± 3	417 ± 3.26	238 ± 2.2	201 ± 2	
C	198 ± 2	166 ± 2.21	205 ± 1.2	182 ± 1	
IL-18					< 0.05
T	804 ± 1.5	2053 ± 1.39	3566 ± 1.82	2009 ± 0.95	
C	2924 ± 1.65	2649 ± 2.65	2840 ± 1.45	2521 ± 1.72	
TNFα					< 0.001
T	30 ± 3	277 ± 2.16	433 ± 3.45	251 ± 3.23	
C	53 ±3	64 ± 2.45	81 ± 3.2	65 ± 3.5	

[a]Abbreviations: T, test; C, control.
[b]All assays were performed in triplicate. Results are means ± SEM from three independent experiments compared with non-stimulated controls, analysed by two-way ANOVA followed by Bonferroni post-test analysis.

Table 3. mRNA-Fold Gene Expression Differences on the Expression of Biomarkers in CP-A Cells Co-Cultured With *C. concisus*[a,b]

Biomarkers	Time, h				P Value
	1	3	5	7	
p53					< 0.01
T	20568 ± 3.35	1684 ± 3.1	952 ± 2.92	672 ± 2.68	
C	2331 ± 2.3	2830 ±3.1	2241 ± 3	2665 ± 3.3	
IL-18					< 0.001
T	387 ± 1.1	401 ± 1.5	1395 ± 2.8	805 ± 1.95	
C	1536 ± 1.65	1758 ± 1.5	1589 ± 1.72	1637 ± 1.52	
TNF-α					< 0.001
T	74 ± 1.52	27 ± 1	51 ± 1.3	568 ± 3.23	
C	3127 ± 2.8	3557 ± 3	3597 ± 3.2	3805 ± 3.5	

[a]Abbreviations: T, test; C, control.
[b]Results are means ± SEM from three independent experiments compared with non-stimulated controls, analysed by two-way ANOVA followed by Bonferroni post-test analysis.

Table 4. mRNA-Fold Gene Expression Differences on the Expression of Biomarkers in CP-D Cells Co-Cultured With *C. concisus*[a,b]

Biomarkers	Time, h				P Value
	1	3	5	7	
p53					< 0.05
T	7494 ± 1.2	7789 ± 1.62	10343 ± 2.32	15836 ± 2.68	
C	15668 ± 5.1	14846 ± 4.7	13905 ± 6	15053 ± 5.3	
IL-18					< 0.001
T	1196 ± 1.65	1335 ± 1.75	1208 ± 3.6	1285 ± 2.95	
C	645 ± 1.6	651 ± 2	726 ± 2.72	745 ± 3.52	
TNF α					< 0.001
T	8.5 ± 1.3	5.8 ± 1	180 ± 3.3	656 ± 4.5	
C	5847 ± 3	7061 ± 3.8	7423 ± 5.2	6718 ± 4.5	

[a]Abbreviations: T, test; C, control.
[b]Results are means ± SEM from three independent experiments compared with non-stimulated controls, analysed by two-way ANOVA followed by Bonferroni post-test analysis.

Table 5. mRNA-Fold Gene Expression Differences on the Expression of Biomarkers in CP-D Cells Co-Cultured With *S. salivarius*[a,b]

Biomarkers	Time, h				P Value
	1	3	5	7	
p53					0.86
T	3206 ± 1.7	3311 ± 1.62	2147 ± 1.32	2550 ± 1.68	
C	2898 ± 6.9	2837 ± 4.7	2822 ± 6	2991 ± 5.3	
IL-18					0.12
T	4.5 ± 1.45	4.75 ± 1.35	4.25 ± 1.2	4.5 ± 1.29	
C	7 ± 2	7.5 ± 2.2	7 ± 1.72	7.5 ± 2.52	
TNF-α					< 0.001
T	275 ± 1.45	415 ± 1.78	490 ± 1.35	650 ± 1.45	
C	4534 ± 2.3	5580 ± 2.8	6120 ± 2.2	4370 ± 2.5	

[a]Abbreviations: T, test; C, control.
[b]Results are means ± SEM from three independent experiments compared with non-stimulated controls, analysed by two-way ANOVA followed by Bonferroni post-test analysis.

5. Discussion

Barrett's oesophagus is recognised as a metaplastic columnar-lined epithelium, which is caused by GORD and predisposes to OA (12). There are various inflammatory responses and gene expression through the progression of BO to OA (20). However, using cell culture model of Barrett's cell lines demonstrated that organism could increase significantly expression of p53, IL-8, and TNF-α in FLO-1 cells. Results showed that considerable influence of *C. concisus* was detected on the expression of p53 in CP-A (non-dysplastic cells) and IL-18 in CP-D (dysplastic cells).

Cellular and molecular alternation has been investigated on the expression of inflammatory cytokines such as IL-1β, TNF-α, IFNϒ, IL-18 and p53 gene. Investigations on IL-18, which is mainly produced by dendritic cells and macrophages, revealed that it influences upregulation of IL-1β, TNF-α and IFNϒ (21). In 2007, Ye et al. (22) presented that high level of IL-18 expression in gastric cancer compared with control group facilitates cancer cell metastasis, and also induces NF-κB pathway to regulate cell proliferation and transformation. The role of IL-18 in induction of different cytokines (IL-1, IL-10, TNF-α) has been documented in gastric and colon cancers and during tumour cell proliferation (23). Detection of pro-inflammatory cytokines (TNF-α, IL-18) in the early stages of BO would be useful to identify the progression of OA, since an association between chronic inflammation and BO has been reported (24). Man et al. (9) reported that *C. concisus* could adhere and invade intestinal Caco2 and HT29 cell lines and release inflammatory cytokines (IL-1β, IL-8 and TNF-α). Similarly, this study showed that bacterium is able to express considerable IL-18 in CP-D and FLO-1 cell lines.

Second selected biomarker was the expression of TNF-α. It acts as a tumour promoter, induces assembling complex receptors, such as TNF-receptor-1 and the Jun kinase (JNK) cascade to control cell survival and death by interplaying between NF-κB and JNK (25). TNF-α is usually activated through multiple intracellular signalling pathways involving NF-kβ, MAPK (mitogen activated protein kinase) and p38 (26, 27). It is expressed at low level in normal squamous epithelium, while its expression increases through the progression of BO signifying a role in the OA evolution (28). This indicates the association between TNF-α, interleukins and NF-kβ. It is known that NF-kβ contributes in upregulation of genes involved in pro-inflammatory cytokine formation, apoptosis blocking and cell proliferation and differentiation.

There is also no activated NF-kβ in normal oesophageal cells; however, it has been detected in BO and OA (21, 29, 30). Activation of NF-kβ via induction of TNF-α and IL-18 through chronic infection enhances the possibility of activation of carcinogenesis sequences. Similarly, stimulation of NF-kβ pathway in Crohn's disease by the effect of *NOD2* gene (31), in gastric cancer *NOD1* gene by the effect of *H. pylori* (32) and in lung cancer *NOD1* by the influence of *Chlamydophila pneumoniea* (33) increases putative role of *C. concisus* in the pathogenesis of BO transformation. Our findings confirmed that microbe could induce increasingly expression of TNF-α on FLO-1 cell line, while its expression on non-dysplastic (CP-A) and dysplastic (CP-D) cell lines were not higher than control in spite of increasing during the time of culture. The impact of *C. concisus* has also been confirmed in Chinese hamster ovary cells regarding cytolytic effect of organism (34) and its influence on the induction of IL-12 and NF-kβ (6).

Another detectable biomarker in Barrett's transformation is p53. Any alternations in the p53 gene would result in various abnormalities and genetical damage. Alternations of p53 gene in BO increase 16-fold risk of OA than those without any abnormalities on p53 (35). However, molecular epidemiology has indicated that the prevalence of positive p53 immunoreactivity in different stages of Barrett's (metaplasia-low dysplasia-high dysplasia-adenocarcinoma) linked to the significant rates of p53 overexpression. These specify that p53 can be induced in early stages of BO via environmental factors, which lead to abnormalities in the cell cycle (36). Subsequently, recognition of induced p53 in different stages of BO movement accounts for the progression of oesophageal cancer. Providing that *C. concisus* induces the operation of signalling pathways to activate p53, it would create genetic instability to establish cellular abnormalities. Evidence indicated an increasing expression of p53 and cell proliferation in correlation with progression of BO between GORD, BO and OA patients, and suppression of p53 in dysplastic stage (27, 37-39). This study showed that *C. concisus* could express significantly p53 in FLO-1 cells, after one hour in CP-A and after seven hours in CP-D; whereas, the major effect was observed in FLO-1 cell line.

This investigation showed that although each of the molecular markers is expressed at different stages of BO transformation, there are close association between their activation and induction of multiple cellular pathways. This study highlighted notable results on the expression of biomarkers compared with the effect of commensal bacterium (*S. salivarius*), which had no considerable impact on the expression of selected biomarkers and can be used in understanding pathogenesis of OA. However, it is early to complete this and there might be a set of unrecognised factors in activating or supressing different biomarkers in BO progression. This study suggested possible pathogenic role of *C. concisus* in initiation or induction of BO to OA as a novel idea to be investigated in further researches.

In conclusion, beside various studies about cellular and molecular mechanisms of BO transformation, we postulated that BO creates a new microenvironment in which BO biofilm specifically *C. concisus* might facilitate induction or exacerbation of BO progression through chronic inflammation.

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Footnotes

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