

## Free fucose is a danger signal to human intestinal epithelial cells

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Fucose is present in foods, and it is a major component of human mucin glycoproteins and glycolipids. L-Fucose can also be found at the terminal position of many cell-surface oligosaccharide ligands that mediate cell-recognition and adhesion-signalling pathways. Mucin fucose can be released through the hydrolytic activity of pathogens and indigenous bacteria, leading to the release of free fucose into the intestinal lumen. The immunomodulating effects of free fucose on intestinal epithelial cells (enterocyte-like Caco-2) were investigated. It was found that the presence of L-fucose up regulated genes and secretion of their encoded proteins that are involved in both the innate and adaptive immune responses, possibly via the toll-like receptor-2 signalling pathway. These include TNFSF5, TNFSF7, TNF- $\alpha$ , IL12, IL17 and IL18. Besides modulating immune reactions in differentiated Caco-2 cells, fucose induced a set of cytokine genes that are involved in the development and proliferation of immune cells. These include the bone morphogenetic proteins (BMP) BMP2, BMP4, IL5, thrombopoietin and erythropoietin. In addition, the up regulated gene expression of fibroblast growth factor-2 may help to promote epithelial cell restitution in conjunction with the enhanced expression of transforming growth factor- $\beta$  mRNA. Since the exogenous fucose was not metabolised by the differentiated Caco-2 cells as a carbon source, the reactions elicited were suggested to be a result of the direct interaction of fucose and differentiated Caco-2 cells. The presence of free fucose may signal the invasion of mucin-hydrolysing microbial cells and breakage of the mucosal barrier. The intestinal epithelial cells respond by up regulation and secretion of cytokines, pre-empting the actual invasion of pathogens.

### Free fucose: Caco-2 cells: Immunomodulation

The gastrointestinal tract is a complex system that actively participates in the protection of the host against aggressions from the external environment. The defence system of the gut comprises three components, namely the microflora, the mucosal barrier and the local immune system, which need to be in permanent contact and continuously communicating with each other<sup>1</sup>. The intestinal epithelial cells act as an essential link in communicating with the immune cells in the underlying mucosa and the microflora in the lumen via the expression of regulatory cytokines.

The gastrointestinal epithelium is covered by a protective mucus gel composed primarily of mucin that is synthesised and secreted by goblet cells<sup>2</sup>. One of the major components of human mucin glycoproteins and glycolipids is L-fucose, a six-carbon deoxyhexose having a galacto-configuration<sup>3</sup>. L-Fucose can also be found at the terminal position of many cell-surface oligosaccharide ligands that mediate cell-recognition and adhesion-signalling pathways<sup>4</sup>. Fucose is also present in certain foods, such as in *Undaria pinnatifida*, a brown seaweed which is one of the richest known sources of fucose<sup>5</sup>.

Research has shown that fucose can be released from mucin through the hydrolytic activity of pathogens such as *Vibrio cholerae*<sup>6</sup> and *Candida albicans*<sup>7</sup> to facilitate the dispersion

of the pathogens along the intestinal tract, and to aid in their penetration of the mucin barrier. Some indigenous bacteria such as those from the genera *Bacteriodes*<sup>1</sup>, *Ruminococcus* and *Bifidobacterium*<sup>8</sup> were found to degrade mucin, leading to the release of free fucose into the intestinal lumen, which was then utilised as an energy source. This would be advantageous for the intestinal colonisation of fucose-utilising bacteria.

However, it is still unclear if free fucose released from intestinal mucin and consumed food through the digestive activity of the intestinal microflora and/or the host plays any role in the microbe–host interaction in the gastrointestinal tract.

The aim of the present study was to determine the immunomodulating effect of fucose in an *in vitro* differentiated Caco-2 cell model.

### Materials and methods

#### *Epithelial cells and culture conditions*

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). This human colon adenocarcinoma cell line was cultured in minimal essential

**Abbreviations:** BMP, bone morphogenetic protein; FGF, fibroblast growth factor; MEM, minimal essential medium; TGF, transforming growth factor; TLR, toll-like receptor.

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medium (MEM; Gibco-BRL, Grand Island, NY, USA) that contained 25 mM-glucose, 20% (v/v) heated inactivated fetal calf serum (Gibco-BRL) and 1% non-essential amino acids (Gibco-BRL). Cells were grown at 37°C in an atmosphere of 5% (v/v) CO<sub>2</sub> in air. The cells were allowed to differentiate into enterocytes by seeding at a density of 1 × 10<sup>5</sup> cells/well in twenty-four-well tissue culture dishes (Nunc, Roskilde, Denmark) and culturing them for 14 d, changing the medium every alternate day.

#### Experimental protocols

Differentiated Caco-2 cells were grown in medium supplemented with or without 0.5% (w/v) L-fucose and incubated for 24 h at 37°C. The culture medium was collected thereafter and stored at -20°C. The cultured Caco-2 cells were also harvested for subsequent RNA extraction.

#### Ribonucleic acid extraction and cDNA array analysis

RNA was isolated from cells in three independent experiments for each treatment using Trizol® reagent (Gibco-RBL). The integrity of the RNA was analysed in Nanodrop® ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). The RNA was processed, labelled and hybridised to the non-radioactive human common cytokines GEArray Q-series Kit (Superarray Inc., Bethesda, MD, USA) according to the cDNA GEArray® Q and S Series Kits user manual (version 8.5; Superarray Inc.). The probed arrays were scanned by flatbed scanner (Amersham Biosciences, Little Chalfont, Bucks, UK) and analysed by MagixScanner software (Amersham Biosciences). The GEArray Expression Analysis Suite was used for calculation of absolute and comparison data.

#### Reverse transcription-polymerase chain reaction

Semi-quantitative reverse transcription was carried out using an oligo(dT) primer, Superscript™ III RT (Invitrogen, Carlsbad, CA, USA) and 1 µg total RNA from at least three independent experiments. The resulting cDNA was amplified using the Platinum® Taq DNA Polymerase kit (Invitrogen) and primers designed. PCR were optimised to the linear amplification

range and run in thirty amplification cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 1 min), followed by a final extension (72°C for 7 min). The PCR products were visualised by agarose gel electrophoresis, and β-actin was used as an endogenous control. Table 1 showed the primer sequences of the various genes assayed for in the RT-PCR analysis.

The bands obtained after gel electrophoresis were analysed using densitometry software (Gene Tools; Syngene, Cambridge, Cambs, UK), a semi-quantitative method to compare gel band intensities. The PCR reactions were normalised to the expression of the gene encoding β-actin. Thereafter, the samples were compared against the control well (without fucose) to obtain the differences in their gene expression.

#### Cytokine protein assay

The quantification of cytokines TNF-α, IL5, IL8, IL12 and IL17 was performed by a Bio-Plex Human 5-Plex Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The amounts of various cytokines in the cell-culture supernatant fractions were quantified in samples obtained from at least three different experiments.

#### High-performance liquid chromatography analysis of fucose

The fucose concentrations in the samples collected were determined by HPLC (Perkin Elmer series 200; Boston, MA, USA). The isocratic HPLC separation was performed using the 300 × 7.8 mm aminex HPX 87C column (Bio-Rad Laboratories). The mobile phase was reverse osmosis water obtained from the Sartorius arium 611VF water purification system (Sartorius, Goettingen, Germany). The system was operated at a flow rate of 0.8 ml/min at 85°C with operating pressure of 390 pounds per square inch (psi). After each sample analysis, the mobile phase was allowed to run for 10 min. Triplicates were done for each condition.

#### Statistical analysis

All statistical analyses in the present study were carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

**Table 1.** Primer sequences used in reverse transcription-polymerase chain reaction analysis

Gene	Direction	Primer sequence	Size (bp)
β-Actin	Forward	5'-GGCGACGAGGCCAGAGCAAGAGAGGCAT-3'	460
	Reverse	5'-CGATTTCCCGCTCGGCCGTGGTGGTGAAGC-3'	
TGF-β1	Forward	5'-TGACAGCAGGGATAACACACT-3'	288
	Reverse	5'-GTAGGGGCAGGGCCCCGAGGCA-3'	
TNF-α	Forward	5'-CAGAGGGAAGAGTTCTTTAG-3'	324
	Reverse	5'-CCTTGGTCTGGTAGGAG-ACG-3'	
TLR2	Forward	5'-GCCAAAGTCTTGATTGATTGG-3'	346
	Reverse	5'-TTGAAGTTCTCCAGCTCCTG-3'	
TRAF6	Forward	5'-GTCGGTACCGCCACCATGAGTCTGCTAACTGTGAA-3'	1600
	Reverse	5'-CGCGGGCCCTATACCCCTGCATCAGTAC-3'	
Mekk3	Forward	5'-CTGTCTGGAAGCTGCCAATC-3'	501
	Reverse	5'-GAAGCAAGTTACGTCCTCCGT-3'	
RelA	Forward	5'-GAAGAAGCGAGACCTGGAG-3'	330
	Reverse	5'-TCCGGAACACAATGGCCAC-3'	

TGF, transforming growth factor; TLR, toll-like receptor; TRAF, TNF receptor-associated factor; Mekk, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; RelA, ppGpp synthetase.

The independent-samples *t* test was used to compare the means for two groups of data. If the significance value for the Levene test was high (typically greater than 0.05), equal variances for both groups were assumed. A low significance value for the *t* test (typically less than 0.05) indicated significant difference between the two group means. In addition, if the CI for the mean difference did not contain zero, this indicated that the difference was significant.

## Results

### Human common cytokine arrays

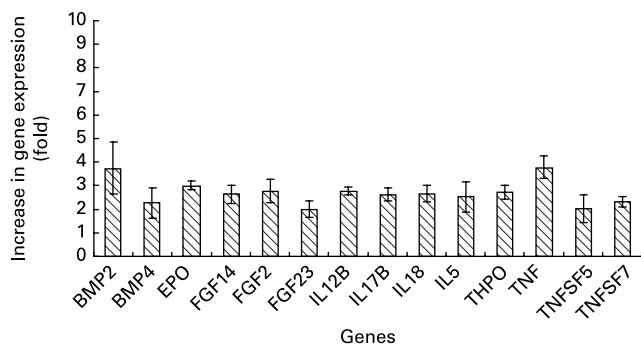
The topmost single layer of intestinal epithelial cells is an integral and essential component of the mucosal immune system. In the present study, we analysed the gene expression in human intestinal epithelial cells (differentiated Caco-2 cells) incubated with or without fucose. After 24 h of incubation, total RNA harvested were hybridised to microarrays containing cDNA of ninety-six selected human cytokine genes.

Almost 70% of the genes represented on the microarray were detected in Caco-2 cell culture grown in normal MEM. The expressions of the genes were considered to be significantly increased or decreased ( $P < 0.05$ ; Student's *t* test) when they showed an average change of at least two-fold in the experimental group (with fucose).

The up regulated genes in the cells treated with MEM supplemented with 0.5% fucose (Fig. 1) were associated with the development and proliferation of immune cells as well as the modulation of both innate and adaptive immune immunity, involved in both cellular and humoral immunity. These genes included TNFSF5, TNFSF7, TNF- $\alpha$ , IL5, IL12, IL17, IL18, bone morphogenetic protein (BMP)-4, thrombopoietin and erythropoietin.

Besides the immune genes found up regulated in the cells incubated with fucose, another set of genes responsible for epithelial cell restitution was found to be up regulated as compared with the control group. These included fibroblast growth factor (FGF)-2 and transforming growth factor (TGF)- $\beta$ .

The four selected cytokine genes, namely IL5, IL12, IL17 and TNF- $\alpha$ , detected by the microarrays were confirmed



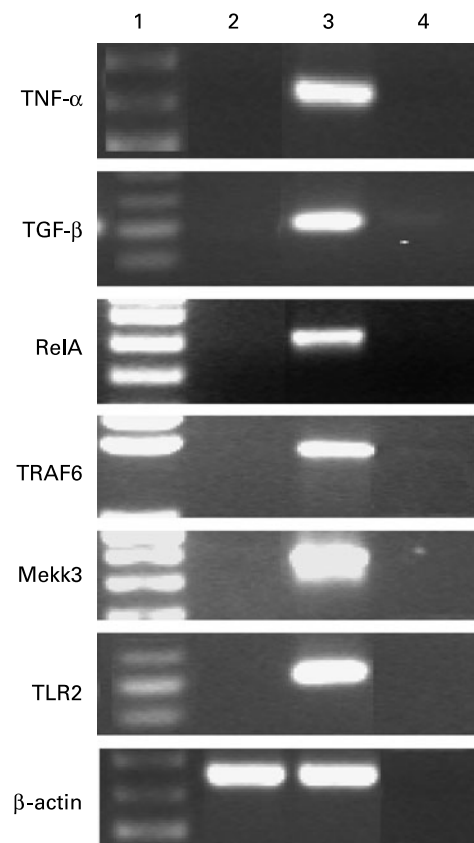
**Fig. 1.** Up regulation of human common cytokine genes in differentiated Caco-2 cells grown in 0.5% fucose compared with cells grown in the absence of fucose. Positive values greater than 2 indicate up regulation by fucose. Values are mean-folds of increase in gene expression from three individual experiments as compared with the control group, with standard deviations represented by vertical bars. BMP, bone morphogenetic protein; EPO, erythropoietin; FGF, fibroblast growth factor; THPO, thrombopoietin.

using the Bio-Plex Human 5-Plex Assay (data shown in the Secretion of cytokines by Caco-2 cells section). Cytokine IL8 was selected for it is an inflammation marker, although there was no significant variation in the gene expression assay. Protein detection assays for BMP, erythropoietin, FGF, IL18 and thrombopoietin are not available. The gene expression could be further confirmed by other techniques such as quantitative real-time PCR, and this will be performed in a future study.

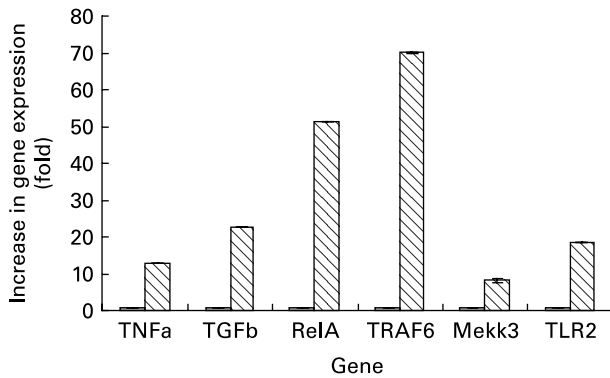
### Reverse transcription-polymerase chain reaction

We first characterised the array of genes associated with the toll-like receptor (TLR)-2 signalling pathway in the control cultures of Caco-2 cells grown in normal MEM. Low levels of mRNA of TLR2, TNF, TGF- $\beta$ , TRAF6, RelA and Mekk3 were found in the control cells (Fig. 2).

When the Caco-2 cells were incubated with fucose, the TLR2 gene expression was up regulated by 10-fold as compared with the control group (Figs. 2 and 3). The expressions of genes downstream of TLR2 in the TLR2 signalling pathway



**Fig. 2.** RT-PCR results. Lane 1 is the TrackIt™ 1 kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA). Lane 2 denotes samples extracted from Caco-2 cells grown in normal minimal essential medium (MEM) (control group). Lane 3 denotes Caco-2 cells grown in MEM supplemented with 0.5% fucose. Lane 4 denotes a negative PCR control done using sterile water instead of the extracted RNA as the template to check for contamination of reagents. RelA, v-rel reticuloendotheliosis viral oncogene homolog A; TRAF, TNF receptor-associated factor; Mekk, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; TLR, toll-like receptor.



**Fig. 3.** Increase of expression of various genes when differentiated Caco-2 cells were incubated with 0.5% fucose (▨), using normal untreated Caco-2 cells as the control (□). Values are mean-folds of increase in gene expression from three independent experiments as compared with the control group. The standard deviations were too minute in value to express. TGF, transforming growth factor; RelA, ppGpp synthetase; TRAF, TNF receptor-associated factor; Mekk, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; TLR, toll-like receptor.

such as TRAF6, Mekk3, RelA, TNF and TGF- $\beta$  were also elevated significantly (Figs. 2 and 3). Incubating Caco-2 cells with medium supplemented with fucose resulted in an approximate 6-fold increase in the mRNA levels for TNF, a 5-fold increase in the Mekk3 mRNA levels, a 50-fold increase in the message levels of RelA, a 6-fold increase in the TNF mRNA levels and a 10-fold increase in the TGF- $\beta$  mRNA levels in comparison with the control cells (Figs. 2 and 3).

#### Secretion of cytokines by Caco-2 cells

The protein endproducts of the detected genes were assayed using the available kit<sup>9</sup>. Concentrations of five selected cytokines, IL5, IL8, IL12, IL17 and TNF- $\alpha$ , in the culture medium were assayed. It is known that some cytokines such as IL8 and TNF- $\alpha$  are expressed and secreted from intestinal epithelial cells<sup>10</sup>. In cell cultures incubated with normal MEM, none of the tested cytokines was detected except for IL8 where a concentration of 13.2 (SD 1.1) pg/ml was detected.

It was found that in the presence of fucose, 0.3 (SD 0.05) pg IL5 protein/ml and 49.9 (SD 3.6) pg IL12 protein/ml were detected (Table 2). This is in agreement with the level of

**Table 2.** Protein levels of selected cytokines secreted\* (Mean values and standard deviations)

Cytokine	Cytokine production (pg/ml)			
	Without fucose		With fucose	
	Mean	SD	Mean	SD
IL5	0	—	0.3	0.05
IL12	0	—	49.9	3.6
IL17	0	—	0	—
IL 8	13.2	1.1	11.0	1.7
TNF- $\alpha$	0	—	1.4	0.1

\* Obtained from at least three different experiments.

gene expression of IL5 and IL12 in the human cytokine gene microarray assay (Fig. 1). This shows that these cytokines were not produced and secreted under normal conditions and that their production and secretion are regulated by fucose.

The gene expressions of IL17 were found to be up regulated in the conditions where the cells were treated with fucose (Fig. 1); however, the protein of IL17 was not detected (Table 2). This might be due to the poor correlation of mRNA expression and protein synthesis. It is also possible that the expression levels of the IL17 cytokine were below the detection limit of the method.

In line with up regulated TNF- $\alpha$  gene expression in the presence of fucose (Fig. 1), 1.37 pg TNF- $\alpha$ /ml was detected in the culture supernatant fraction (Table 2).

Since Caco-2 cells originate from colon adenocarcinoma, it is not surprising to detect the pro-inflammatory cytokine IL8 protein in differentiated Caco-2 cells grown in normal media (Table 2). The secreted protein level of IL8 detected for the differentiated Caco-2 cells incubated in media supplemented with fucose was significantly lower as compared with the control group (Table 2).

#### Fucose uptake by Caco-2 cells

After 24 h of incubation with media supplemented with 5 mg fucose/ml, the average concentration of free fucose in the supernatant fractions collected was 4.7 mg/ml. This suggested that the differentiated Caco-2 cells did not use exogenous fucose significantly. Hence, the immune responses of differentiated Caco-2 cells observed in the presence of fucose were a result of the interaction of free fucose with the Caco-2 cells rather than the cells' metabolism of the sugar.

#### Discussion

When differentiated enterocyte-like Caco-2 cells were treated with fucose, the expression of fourteen cytokine genes was found increased and none of the genes measured decreased their expression (Fig. 1). From the results obtained from the gene expression studies, fucose seems to play a role in activating genes involving in inflammatory responses. The pro-inflammatory genes up regulated include those for TNF- $\alpha$ , IL12, TNFSF5, TNFSF7, IL17 and IL18.

TNFSF5 (CD40L) regulates B cell function by engaging CD40 on the B cell surface<sup>11</sup> and this interaction helps to drive B cells into the cell cycle<sup>12</sup>. This signal is essential for the germinal centre development and antibody responses to T-cell dependent antigens<sup>12</sup>.

The ligand TNFSF7 induces the proliferation of co-stimulated T cells, enhances the generation of cytolytic T cells, and contributes to T cell activation<sup>13</sup>. IL17B codes for the T-cell-derived cytokine that shares sequence similarity to IL17. It was reported that IL17B is capable of inducing the release of TNF- $\alpha$  and IL1B from a monocytic cell line<sup>14</sup>.

IL18 codes for the pro-inflammatory cytokine that has interferon- $\gamma$ - and TNF- $\alpha$ -inducing activity that contributes to the development of various inflammatory diseases<sup>15</sup>. It has been reported that the combinatorial effect of IL12 and IL18 inhibits the production of IgE by the induction of interferon- $\gamma$

production by activated B cells, which may present a unique approach to the treatment of allergy<sup>16</sup>.

Another interesting observation noted was that the gene expression of TLR2 was elevated 10-fold in comparison with the control group. The expressions of genes downstream of TLR2 in the TLR2-signalling pathway such as TRAF6, Mekk3 and RelA were also elevated. This suggests that in a condition where fucose exceeds that of the physiological concentration, the intestinal epithelial cells may be primed for inflammatory reactions via the TLR2-signalling pathway (Figs. 2 and 3).

NF- $\kappa$ B regulates a wide variety of genes encoding for cytokines such as IL1, IL2, IL6, IL8, IL12, TNF- $\alpha$ , lymphotoxin- $\alpha$ , lymphotoxin- $\beta$  and granulocyte-macrophage colony-stimulating factor<sup>17</sup>. As mentioned earlier, both the IL12 and TNF- $\alpha$  gene expressions were found to be up regulated, suggesting that fucose may have evoked the transcription of target cytokine genes of NF- $\kappa$ B via the TLR2 pathway. Furthermore, the up regulated gene expression of TNF- $\alpha$  detected in both the microarrays and RT-PCR analysis was in agreement with the detected level of TNF- $\alpha$  protein (Table 1). IL12 may serve as an essential inducer of T helper-1 cell development<sup>12</sup>. Similarly, TNF- $\alpha$  activates immune cells such as macrophages and granulocytes<sup>12</sup>.

It appears that fucose modulates both innate and adaptive immune immunity, and is involved in both the cellular and humoral immunity of the latter.

Besides modulating the immune reactions in differentiated Caco-2 cells, fucose induced a set of cytokine genes that are involved in the development and proliferation of immune cells. This set of genes includes BMP2, BMP4, IL5, thrombopoietin and erythropoietin (Fig. 1).

The BMP group consists of BMP2 and BMP4, whose gene expressions were elevated approximately 3- and 2-fold respectively as compared with the control group (Fig. 1). Both BMP2 and BMP4 are members of the TGF- $\beta$  superfamily. Of the two BMP genes found up regulated, BMP4 can regulate the development and proliferation of human haematopoietic stem cells by acting as a survival factor, which aids in preserving stem cell function under conditions that normally lead to stem cell loss<sup>18</sup>. Undifferentiated human haematopoietic stem cells are crucial in the immune system since they differentiate under the influence of the microenvironment, such as cell-to-cell interactions and presence of soluble or membrane-bound cytokines, to give rise to many cells that are involved in the immune response<sup>12</sup>.

IL5 acts as a growth and differentiation factor for both B cells and eosinophils. This cytokine is a main regulator of eosinopoiesis, eosinophil maturation and activation<sup>19</sup>.

Thrombopoietin is a humoral growth factor that is necessary for megakaryocyte proliferation and maturation, as well as for thrombopoiesis<sup>20</sup>.

The colony-stimulating factor erythropoietin plays a role in the process of erythropoiesis<sup>21</sup>. Erythropoietin gene expression has been found to induce the expression and secretion of TNF- $\alpha$ , which plays a key role in the inflammatory response<sup>22</sup>.

In addition to the cytokine genes that are involved in the development and proliferation of immune cells, members of the FGF family were found up regulated by fucose in the present study. FGF family peptides possess broad mitogenic and

cell-survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumour growth and invasion<sup>23</sup>. Besides having an effect on the fibroblast cells, FGF especially the acidic FGF (FGF2) can also exert their effects on gastrointestinal cells. The up regulated gene expression of FGF2 may help to promote epithelial cell restitution in conjunction with the enhanced expression of TGF- $\beta$  mRNA (Figs. 2 and 3) since the process of intestinal epithelial cell turnover is highly dynamic, occurring every 24–96 h among different mammalian species<sup>24</sup>.

It was noted that the exogenous source of fucose was not metabolised by the differentiated Caco-2 cells. Hence it is the direct interaction of fucose with differentiated Caco-2 cells that brought about the modulation of inflammatory reactions, the regulation of the development and proliferation of the haematopoietic stem cells as well as the possible restitution of the intestinal epithelial cells in the *in vitro* model.

Fucose is reported to modulate the attachment of enteric pathogens such as *V. cholerae* on the intestinal surface<sup>25</sup>. Earlier studies suggested that fucose-containing structures on eukaryotic cells may function as receptors for vibrio adhesion and therefore may be important determinant of host susceptibility<sup>26</sup>. *V. cholerae* produces the enzyme neuraminidase that attacks the intestinal glycoproteins and gangliosides, thereby unmasking receptor sites for cholera toxin<sup>27</sup>. Fucose is a major component of mucus of the gut; a sudden increase in the concentration of fucose in the gut lumen may be indicative of a breach of the intestinal barrier of mucus by enteroinvasive pathogens. We would like to propose that free fucose serves as a danger signal for the first line of defence in the intestine leading to up regulation of the expression of cytokine genes involved in the development of immune cells as well as induction of the adaptive and innate immunity. It is known that some intestinal indigenous bacteria are able to degrade mucin, releasing fucose<sup>1,8</sup>, and commensal bacteria have been reported to stimulate host immune reactions<sup>28</sup>. It is likely that the fucose danger-signalling mechanism is not specific for pathogens, but a response to the breach of the mucosal barrier.

In conclusion, free fucose appears to play the role of a mediator in the modulation of immune responses in human intestinal epithelium from cDNA studies. The presence of free fucose in the human intestinal tract may signal the invasion of mucin-hydrolysing microbes and breakage of the mucosal barrier. The intestinal epithelial cells respond by up regulation and secretion of cytokines, pre-empting the actual invasion of pathogens. This signalling mechanism may also be used by commensal (probiotic) bacteria in the enhancement of host immune responses and in the maintenance of mucosal barrier integrity.

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