Blastocystis hominis and colorectal cancer

Blastocystis hominis (B. hominis) is the most common unicellular protozoan parasite that is found in the human gastrointestinal tract (Windsor et al. 2002). The prevalence of B. hominis in both developed and developing countries is reported to be up to 10% and 50% respectively (Wong et al. 2008; Stenzel and Boreham. 1996). On the other hand, B. hominis shows a diverse morphologies which include vacuolar, granular, amoeboid and cyst forms (Zierdt. 1988). Furthermore, B. hominis isolates from human and animals have been reported to exhibit an extensive genetic and karyotypic heterogeneity (Parkar et al. 2010; Stensvold et al. 2009; Tan et al. 2009; Dogruman-Al et al. 2009; Abe. 2004; Yoshikawa et al. 2004).

Although many researchers have attempted to identify the pathogenesis of B. hominis in human hosts, however the pathogenic potential of B. hominis has remained controversial as it is present in both symptomatic and asymptomatic individual (Tan. 2008; Garcia. 2007; Tan et al. 2002). The gastrointestinal symptoms that are associated with B. hominis infection include diarrhea, vomiting, anorexia, flatulence, abdominal discomfort and other associated non-specific symptoms. To date, experiments have shown that rats that are inoculated with human B. hominis isolate have demonstrated an elevation in oxidative damage (Chandramathi et al. 2009).

Many studies have been done on the genetic and molecular characterization of B. hominis isolates derived from human as well as animal. Recent molecular characterization of Blastocystis isolates suggested that there are 13 subtypes (ST1-ST13) which have been isolated from mammalian, reptilian, avian and amphibian hosts (U. Parkar et al. 2010; Stensvold et al. 2007a; Noël et al. 2005, 2003; Yoshikawa et al. 2004b). Recently, many

studies have been done to determine the pathogenic potential of different Blastocystis subtypes in human hosts by genotypic analysis as well as molecular characterization of B. hominis isolated from symptomatic and asymptomatic individuals (Dominguez-Márquez et al. 2009; Eroglu et al. 2009; Jones et al. 2009; Hussein et al. 2008; Tan et al. 2008). Nevertheless, only limited numbers of studies have shown correlation between the different B. hominis subtypes on the pathogenesis in human hosts (Hussein et al. 2008).

Infectious agents such as bacteria, virus and parasites, often have an oncogenic potential. The International Agency on Research of Cancer (IARC) has estimated that 16% of cancer worldwide is caused by infection, including parasites. However, there has been no research done on the association between B. hominis infection and colorectal cancer. A recent study has suggested that B. hominis may possess the ability to induce the growth of colorectal cancer cell lines by inhibiting the apoptotic effect of colon cancer cells. Furthermore, the antigens that are isolated from B. hominis were postulated to be able to promote the proliferation of cancer cells via down-regulation of host immune cellular responses (Chandramathi et al. 2010).

In general, inflammation is activated by a variety of stimuli such as trauma, bacterial/viral/parasitic infections, endotoxemia and heating (Ley, 2001; Hart, 2002). Inflammation caused by infectious agents such as parasites will cause an increase in the production of reactive oxygen species (ROS) such as nitric oxide, hydrogen peroxide and superoxide as a consequence of cell mediated phagocyte dependent immune response (Rosen et al. 1995). A persistent and chronic inflammatory response can be detrimental to human

host as it can produce a chronic damage by releasing a variety of pro-inflammatory and anti-inflammatory cytokines which then leads to mutagenesis, carcinogenesis, neurodegenerative disorder, inflammatory bowel syndrome and atherosclerosis (Kühn et al. 1993; Perry et al. 1998; Ludewig et al. 2002; Shacter et al. 2002). Several studies have shown correlation between the inflammation that is caused by infectious agents such as parasites and the development of cancer in human (Fitzpatrick. 2001). Thus, it is important for us to evaluate the immunomodulation, cytopathic and cellular cytokines responses as a result of B. hominis infection especially in colorectal carcinomas. Since B. hominis is often present in most stool cultures, it is pertinent to investigate the association between B. hominis infection and the development of colorectal cancer in the gastrointestinal tract of infected host system.

B. hominis isolated from an asymptomatic individual could facilitate the proliferation and growth of cancer cells and has the potential to down-regulate the host immune response (Chandramathi et al. 2010). However, only limited numbers of studies have shown the cytopathic effects and cellular cytokine responses of Blastocystis hominis infection (Puthia et al. 2008; Long et al. 2001; Walderich et al. 1998). These studies only investigated on Interleukin 8 and Granulocyte-macrophage colony stimulating factor (GM-CSF) which are pro-inflammatory cytokines.

We hypothesized that B. hominis infection in human host system has a potential carcinogenic effect and could influence the growth of colorectal cancer cells especially in colorectal cancer patients and that the B. hominis subtypes may exert varying degree of pathogenecity. Therefore, in this

study, the differences between the effects of solubilized antigen of B. hominis from both symptomatic and asymptomatic isolates on the cell viability in the peripheral blood mononuclear cells (PBMCs, which represent the immune cells) as well as colorectal cancer cell line was evaluated. Besides that, the gene expression of cytokine, apoptotic mediators, and nuclear transcriptional factors in response to the symptomatic and asymptomatic B. hominis antigen in both PBMCs and colorectal cancer cell was compared. The knowledge and understanding in the association between Blastocystis hominis infection and colorectal cancer as well as the role of various cytokines involved in the tumour progression may provide an insight into prevention and/or development of new immune-therapeutical strategies to combat colorectal cancer.

OBJECTIVE

The objectives of the present study are:-

To study the effects of solubilized antigen of B. hominis from symptomatic and asymptomatic individual isolates on viability of both the peripheral blood mononuclear cells (PBMCs, which represent the normal immune cells) as well as colorectal cancer cells (HCT 116, colorectal cancer cell line).

To investigate the gene expression of cytokine, transcriptional factors and apoptotic mediators such as interleukin 6, interleukin 8, tumour necrosis factor-α, interferon gamma, nuclear factor kappa light chain enhancer of activated B cells, cathepsin B, transforming growth factor-β, and protein 53 upon exposure to B. hominis antigen in both the PBMCs and colorectal cancer cells

MATERIALS AND METHODS

Sample collection and axenization of Blastocystis hominis

B. hominis was isolated from stool samples of symptomatic as well as asymptomatic individuals. The B. hominis cysts were then isolated from feacal sample using the Ficoll-Paque Technique according to Zaman and Khan (1994). The harvested cysts of B. hominis from both the symptomatic and asymptomatic individual isolates were washed in sterile phosphate-buffered saline (PBS). The washed harvested cysts were cultured in Jones medium, supplemented with 10% of heat-inactivated horse serum and incubated at 37°C in CO2 incubator (Suresh and Smith. 2004). Axenization was conducted after the isolates have been incubated for 2 to 3 days.

Isolation of solubilized antigen from symptomatic and asymptomatic cultures

The axenic B. hominis species were collected using Ficoll-Paque density gradient centrifugation method and the harvested parasites were resuspended in basal Jones medium without the addition of heat-inactivated horse serum. Then, the harvested organisms were lysed by sonication method and the homogenate was incubated at 4°C, overnight. The homogenate was centrifuged at 13000xg for 15 minutes at 4°C after the overnight incubation. The supernatant which contains the solubilized antigen of B. hominis was filter-sterilized and the protein concentration of the antigen was determined using Bradford assay (Bio-Rad, USA).

Subtyping of Blastocystis isolates from symptomatic and asymptomatic cultures

Genomic DNA Extraction: The genomic DNA of symptomatic and asymptomatic isolates of Blastocystis hominis were extracted from the 3 to 4

days old culture with QIAmp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol.

DNA Purity and Concentration: The genomic DNA yields were estimated using measurement of absorbance at 260nm and 280nm using a spectrophotometer. The ratio of absorbance at 260nm to 280nm determines the purity of the genomic DNA yielded. According to the manufacturers' protocol, the ratio of 1. 7-1. 9 indicates that the DNA yields are pure.

PCR Typing by STS Primers: The genotype of B. hominis was determined by using PCR amplification with subtype-specific sequence tagged site (STS) primers (SB83, SB155, SB227, SB332, SB340, SB336, and SB337). The PCR reaction mixtures (20 µl of total volume) consisted of PCR buffer, 2. 5 U/µl of Taq DNA polymerase (Fermantas, SB38), 1. 5 mM MgCl2, 1. 25 µM of dNTPs (Fermantas, RO191), 0. 5 pmol of forward primer, 0. 5 pmol of reverse primer, and 1. 0 µl of the DNA samples. In addition, the PCR conditions were set as follows: one cycle denaturing at 94°C for 3 min, 30 cycles including denaturing at 94°C for 30 seconds, annealing at 56. 3°C for 30 seconds, extending at 72°C for 60 s, , and additional cycle with a 10 min chain elongation at 72°C. The PCR products obtained were then electrophoresed in 1. 5% agarose gel with 1X Tris-boric-EDTA (TBE) buffer. The fragments of the DNA were visualized using UV illuminator under UV light. The fragment sizes of the genomic DNA were confirmed with bands of a DNA ladder (50-1, 000 bp DNA markers, Fermentas).

Cultivation and collection of HCT116

Human colorectal carcinoma cell line, HCT116, was cultured in 25 cm^3 culture flask containing 5 ml of RPMI 1640 growth medium, supplemented with 5% of FBS, 2mM L-glutamine, 100 U/ml penicillin-streptomycin, and 2. 5 µg/ml fungizone. The HCT116 cell line was then incubated in a CO2 incubator set at 100% humidity, atmosphere containing 5% of CO2 and a temperature of 37°C. Prior to the introduction of antigen from Blastocystis hominis isolates, the cells were harvested from the substratum of the culture flask by using 0. 25% trypsin-EDTA. The harvested cells were then washed with phosphate-buffered saline (PBS) thrice and resuspended in RPMI 1640 growth medium before introducing the solubilized antigen of Blastocystis hominis from symptomatic and asymptomatic isolates.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were collected from fresh human blood sample (12 ml) in sterile EDTA tubes. The PBMCs were then isolated from the blood sample by using Histopaque®-1077 (Sigma-Aldrich, USA) according to the density gradient centrifugation method (Boyum. 1974). The isolated PBMCs were then washed with PBS thrice and resuspended in 5 ml of RPMI 1640 growth medium, supplemented with 10% of FBS, 2mM L-glutamine, 100 U/ml penicillin-streptomycin, and 2. 5 µg/ml fungizone in a 25 cm^3 culture flask prior to the introduction of solubilized antigen of Blastocystis hominis isolates.

Introduction of the solubilized symptomatic and asymptomatic derived Blasto antigen into PBMCs and colorectal cancer cell line, HCT116

Harvested HCT116 cells (1 x 103 cells per well) in 100 µl of RPMI growth medium with 5% FBS and freshly isolated PBMCs (5 x 104 cells per well) in 100 µl of RPMI growth medium with 10% FBS were seeded into 96 well plates. After the overnight incubation in a CO2 incubator containing 5% CO2 at 37°C, Blasto-antigen of B. hominis from symptomatic and asymptomatic individuals at final concentration ranging from 0. 001 to 10µg/ml was added to each well containing the PBMCs and HCT116 cells and were further incubated for 48 hours. Then, the cell proliferation/viability was measured using the MTT assay (Mosmann. 1983).

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (rtRT-PCR)

Concentration of symptomatic and asymptomatic Blasto-antigen that yields the optimal proliferation and inhibition in the preliminary tests (MTT assay) was used to introduce into each culture flask containing PBMCs and HCT 116 cells respectively. For controls, PBS was introduced into both types of cells. After 48 hours of incubation, the RNA was isolated from the PBMCs and HCT 116 cells using Ambion RNAqueous Micro Kit (Ambion, CA, USA). The purified RNA obtained was then used to synthesize complementary DNA (cDNA) using High-Capacity RNA-to-cDNA kit (Applied Biosystems, USA) by the PCR method. Finally, Real-time reverse transcription PCR analysis was performed using inventoried primers (TaqMan® Gene Expression Assays, Applied Biosystems). In this study, the genes of interest were IL-6, IL-8, tumour necrosis factor alpha (TNF-α), nuclear factor kappa light chain enhancer of

activated B cells (NF-κB), interferon gamma (IFN-γ), protein 53 (p53), transforming growth factor beta (TGF-β), and cathepsin B (CTSB). The PCR reaction will be prepared according to the protocol that is generated by StepOneTM Software v2. 0.

Statistical Analysis

In order to analyze the real-time RT-PCR gene expressions, the âˆ†CT value of the treated samples with symptomatic and asymptomatic Blasto-antigen was compared against the non-treated sample (control with PBS) using the Student’s t test. A P value of 0. 05 was considered to be the minimum threshold of significance.

RESULTS AND DISCUSSIONS

In the present study, the symptomatic and asymptomatic isolates were genotyped by PCR amplification using the well-known seven sets of STS primers. The extracted DNA of symptomatic isolate showed positive amplification with SB83 and thus determined as subtype 1 (Yoshikawa et al. 2004). Nevertheless, the extracted DNA of asymptomatic isolate did not show any positive amplification with all the seven sets of STS primers that was used in this study. Hence, it is postulated that the asymptomatic isolate may be subtype 8 to 13 which was not amplified by the STS primers used in this study (Dominguez-Márquez et al. 2009; Eroglu et al. 2009; Jones et al. 2009; Hussein et al. 2008; Tan et al. 2008). Molecular phylogenetic analysis need to be carried out in order to further characterized the subtype of asymptomatic isolate used in this study (Noel et al. 2005; Stensvold et al. 2007; Yoshikawa et al. 2007).

In this study, the Blasto-Ag isolated from both symptomatic as well as asymptomatic isolates have caused an inhibition of PBMC cells proliferation which leads to the speculation that the Blasto-Ag may have caused apoptosis in the immune cells to prevent the propagation of immune cells to combat with the Blastocystis infection. The symptomatic isolate has caused a greater inhibition of PBMCs as compare to asymptomatic isolates. However, the Blasto-Ag mediated PBMCs cell inhibition is contrast to our previous report (Chandramathi et al. 2010). Our previous study showed that Blasto-Ag caused stimulation of PBMCs cell proliferation (Chandramathi et al. 2010). However, the molecular genotyping of the B. hominis isolates used in that study was not carried out. Past studies have reported that there may be a correlation between different B. hominis subtypes on the pathogenesis in human hosts (Dominguez-Márquez et al. 2009; Eroglu et al. 2009; Jones et al. 2009; Hussein et al. 2008; Tan et al. 2008). This contrasting result observed in the cell viability of PBMCs may suggest the different subtypes of B. hominis may give different effects on the immune cells. Hence, further investigation is required to investigate the potential pathogenesis of different B. hominis subtypes.

In the current study, increase in cell proliferation has been observed in colorectal carcinoma cell line, HCT116 cells upon exposure to antigen from symptomatic as well as asymptomatic isolates. HCT116 cells have shown significantly higher increase in cell proliferation stimulated by symptomatic Blasto-Ag as compared to asymptomatic antigen. The increase in the cell proliferation may further suggest that Blastocystis infection could facilitate the growth of colorectal cancer cells (Chandramathi et al. 2010). It is evident

from the present study that symptomatic Blasto-Ag is more pathogenic as compared to asymptomatic Blasto-Ag. Hence, it may be essential to treat the Blastocystis infection in symptomatic colorectal cancer patients for better prognosis.

On the other hand, a diverse pattern of cytokine, nuclear transcription factor as well as apoptotic gene expressions were observed upon the stimulation of HCT116 cells and PBMCs by symptomatic and asymptomatic derived Blasto-Ag. In the present study a noticeable up-regulation of Th1 (IFN-γ and TNF-α) and Th2 (IL6, IL8 and TGF-β) cytokines was observed in HCT116 following exposure to symptomatic Blasto-Ag may lead to the speculation that B. hominis has activated the cellular and humoral immune responses in clearing the Blastocystis infection. This is accordance to the previous study that extracellular parasites such as B. hominis would be more effective in counteracted by a combination of Th1 and Th2 cytokines (Daugelat et al. 1996). Nevertheless, a more significant up-regulation of Th2 cytokines as compared to Th1 cytokines observed in HCT116 upon exposure to symptomatic and asymptomatic Blasto-Ag may lead to the postulation that symptomatic Blasto-Ag may resulting in a Th2-dominated responses and has the potential in weakening the cellular immune response, allowing the progression and growth of an existing tumour cells. On the other hand, HCT116 exposed to symptomatic Blasto-Ag has resulted in a more noticeable up-regulation of Th2 cytokines then asymptomatic Blasto-Ag which may lead to the speculation that asymptomatic Blasto-Ag is less pathogenic than symptomatic derived Blasto-Ag. Furthermore, the up-regulation of TGF-β in the colorectal cancer cells has the potential role of anti-inflammatory and

resulting in host immunosuppression by inhibiting the cell mediated immune responses against the tumour cells (Zou, 2005; Seruga et al. 2008).

In addition, it is widely-accepted that humoral immunity is activated by extracellular microbes such as bacteria (Romagnani, 1996). B. hominis, being an extracellular allergen, has triggered the activation of host humoral immune responses which is evident by the significant up-regulation of Th2 cytokines in the PBMCs upon exposure to symptomatic and asymptomatic Blasto-Ag. Moreover, this results can be supported by the recent finding which shown an elevation of IL6 and IL8 level in the monocytes of healthy volunteers exposed to Leishmania infection (Menezes et al. 2008). In spite of that, an interesting finding has been observed in PBMCs stimulated by symptomatic and asymptomatic Blasto-Ag, besides activating the humoral immune responses, PBMCs exposed by Blasto-Ag has also triggered the activation of cellular mediated immune response characterized by Th1 cytokines. As described earlier, extracellular parasites may be more effective counteracted by a combination of Th1 and Th2 cytokines mediated immune responses (Daugelat et al. 1996). However, the activation of cellular mediated immune responses which is reflected by a significant up-regulation of TNF-α and IFN-γ may lead to the speculation that Blasto-Ag isolated from symptomatic as well as asymptomatic individual has the ability in causing extensive inflammatory damage in the host tissue as a result of macrophage mediated responses towards the parasitic infections.

In addition, an up-regulation of NF-κB and pro-inflammatory cytokines IL6 and IL8 was observed in HCT116 stimulated by symptomatic Blasto-Ag. Furthermore, these findings were also observed in PBMCs stimulated by

symptomatic and asymptomatic Blasto-Ag. Nevertheless, HCT116 exposed to asymptomatic Blasto-Ag has exhibited an insignificant down-regulation of NF-ÐºB gene expression which then leads to a significant lower expression in IL6 and IL8 as compared to HCT116 stimulated by symptomatic Blasto-Ag. Although the previous reports by Chandramathi et al. 2010 has stated the potential role of asymptomatic Blasto-Ag in causing the up-regulation of NF-ÐºB and pro-inflammatory cytokines IL6 and IL8, however, the similar trend was not observed in the current study. As described earlier, different Blastocystis subtypes may have different effects on HCT116 cells as well as causing different gene expression profile in HCT116. To further characterize the gene expression profiles as well as pathogenic potential of each subtype on HCT116 and PBMCs, more samples on different subtypes need to be recruited for the future study.

Apart from this, NF-ÐºB is also related to the apoptotic mediator genes namely p53 and CTSB. NF-ÐºB has been postulated to play a role in inhibiting the apoptosis; hence it is hypothesized that the down-regulation of NF-ÐºB gene expression will lead to the up-regulation of p53 and vice versa (Baldwin, 2001; Chwieralski et al. 2006). Although the recent study has suggested that CTSB may contribute to the cell apoptosis, however the studies have only reported on CTSB-mediated apoptosis in breast cancer cells as well as hepatocytes apoptosis in fulminant hepatic failure (Sandes et al, 2007; Yan et al. 2009). Nevertheless, the possibility of CTSB participation in the apoptosis of colorectal cancer cells has not been investigated. Moreover, there are a number of studies have shown that over expression of CTSB mRNA and elevation of its protein activity have been associated with

the invasive and metastasis properties of various cancers (Campo et al. 1994; Yan et al, 1998; Hirai et al. 1999; Sanjeeva et al. 2001). The similar findings were observed in the current study where the HCT116 cells stimulated by symptomatic as well as asymptomatic Blasto-Ag have caused a significant up-regulation of CTSB. The over expression of CTSB in HCT116 cells stimulated by symptomatic and asymptomatic Blasto-Ag may implicate the potential of Blasto-Ag in causing the invasive and metastasis of colorectal cancer (Campo et al. 1994; Yan et al. 1998; Dora et al. 2003).

In the present study, the insignificant down-regulation of p53 gene expressions observed in PBMCs may indicates that the symptomatic and asymptomatic Blasto-Ag may have prevented the PBMC cells to undergo the apoptosis process. The observation of p53 gene expressions obtained was contrary to the expectation. This may be due to the latent effects of the host immunity to down-regulate the apoptotic mediator gene in order to combat with the invasive B. hominis as well as to prevent persistent inflammation which can lead to carcinogenesis. Although the down-regulation pattern was observed, it does not causing a significant effect (0. 95 and 0. 96 fold decrease respectively).

In contrast to PBMCs, stimulation of symptomatic Blasto-Ag in HCT116 has insignificantly down-regulated the gene expressions of p53. The insignificant down-regulation observed in HCT116 stimulated by symptomatic Blasto-Ag may be explained that it could be the reason that HCT116 cells to have low level of NF-ÐºB gene expression level (1. 34 fold increase). Nevertheless, the down-regulation observed may further suggest that symptomatic Blasto-Ag may has enhance the proliferation and progression of existing tumour

besides causing an extensive release of pro-inflammatory cytokines. On the other hand, an interesting finding was observed in HCT116 exposed to asymptomatic Blasto-Ag, instead of causing down-regulation of p53 gene expression, asymptomatic Blasto-Ag has caused an up-regulation of p53 (1. 09 fold increase) in HCT116.

The findings and observations obtained from the current study are still far to characterize the mechanism of the potential pathogenic role of B. hominis in PBMCs as well as HCT116 cells. However, the current study was able to give an overview of the potential pathogenesis of symptomatic and asymptomatic derived Blasto-Ag in HCT116 and PBMCs. Moreover, the findings also suggest that symptomatic derived Blasto-Ag is more pathogenic as compare to asymptomatic Blasto-Ag. More studies still need to be done on the association of this emerging parasite on the association with the colorectal cancer.

CONCLUSION

The MTT cell proliferative assay and gene expression profile in this study has shown that the solubilized antigen isolated from symptomatic and asymptomatic individuals, at a particular concentration, could facilitate the proliferation and growth of colorectal carcinoma while having the ability to induce apoptosis on PBMCs immune cells. Moreover, the cell proliferative assay also successfully reported that antigen isolated from symptomatic individual is more pathogenic as compare to asymptomatic isolates, as it causes a significantly higher increase in cell proliferation of HCT116 colorectal carcinoma cell lines and a significantly higher increase in inhibition of PBMCs immune cells. Moreover, symptomatic isolate of B. hominis has

exhibited a more evident increase in the gene expression of Th1 and Th2 cyokines. Hence, there is a vital need to screen colorectal cancer patients for B. hominis infection.