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Trichomonas vaginalis adherence mediates differential gene expression in human vaginal epithelial cells

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Summary

***Trichomonas vaginalis*, an ancient protist, colonizes the vaginal mucosa causing trichomonosis, a vaginitis that sometimes leads to severe health complications. Preparatory to colonization of the vagina is the adhesion to vaginal epithelial cells (VECs) by trichomonads. We hypothesized that VECs alter the gene expression to form a complex signalling cascade in response to trichomonal adherence. In order to identify the genes that are upregulated, we constructed a subtraction cDNA library after contact with parasites that is enriched for differentially expressed genes from the immortalized MS-74 VECs. Sixty cDNA clones were sequenced and to our knowledge for the first time, differentially regulated genes were identified in response to early trichomonal infection. The identified genes were found to encode functional proteins with specific functions associated with cell structure maintenance and extracellular matrix components, proinflammatory molecules and apoptosis. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed expression of selected genes. Further, cyclooxygenase 2 (COX-2) protein expression was analysed using Western blot and immunofluorescence assays. Data suggest that p38 mitogen-activated protein (MAP) kinase and tyrosine kinases play a role in COX-2 induction. Finally, *T. vaginalis* and *Tritrichomonas foetus* but not *Pentatrichomonas hominis* induce expression of COX-2. This is a first attempt at elucidating the basis of interaction of trichomonads with host cells and the corresponding host responses triggered by the parasites.**

Introduction

Trichomonas vaginalis is the causal agent of trichomonosis, the number one non-viral sexually transmitted disease (STD) worldwide (World Health Organization, 1995), and infection with this parasite may bring about serious consequences to women's health and occasionally in men. Women with symptoms will experience a foul-smelling discharge, abdominal pain, irritation and severe discomfort. In women, complications are associated with adverse pregnancy outcomes (Cotch *et al.*, 1997), preterm birth (Minkoff *et al.*, 1984), greater risk of tubal infertility (El-Shazly *et al.*, 2001), atypical pelvic inflammatory disease (Moodley *et al.*, 2002), amplified HIV transmission (Sorvillo *et al.*, 2001) and increased risk of cervical cancer (Viikki *et al.*, 2000). Some infected men present a non-chlamydial, non-gonococcal urethritis (Krieger *et al.*, 1993) concomitant with increased risk of facilitating HIV transmission (Hobbs *et al.*, 1999). Interestingly, however, a large proportion of men are asymptomatic to trichomonal infections, and the infection is believed to be self-limiting (Krieger and Riley, 2002). For women, an annual incidence in the USA alone is estimated at 8 million new cases of trichomonosis (Weinstock *et al.*, 2004). The complications from trichomonosis coupled with the enhanced risk factor for HIV transmission and predisposition to cervical cancer means there is a need to understand the mechanisms of pathogenesis and the host responses to infection by *T. vaginalis*.

Adhesion of *T. vaginalis* to vaginal epithelial cells (VECs) plays an important role in pathogenesis of trichomonosis (Arroyo *et al.*, 1992). Four different parasite surface proteins mediate adherence, and the adhesins are upregulated during attachment to VECs (Arroyo *et al.*, 1993; Garcia *et al.*, 2003). While overall immune responses during trichomonosis are largely unknown, high levels of interleukin-8 (IL-8) and leukotreine B4 (LTB4) have been found in the vaginal discharges from patients with symptomatic trichomonosis (Shaio *et al.*, 1994; 1995; Shaio and Lin, 1995). There are also reports of IL-8 being produced by human neutrophils (Ryu *et al.*, 2004) and human monocytes (Shaio *et al.*, 1995) in response to *T. vaginalis* stimulation using *in vitro* assay systems. Further, *in vitro* studies have revealed that IL-8 production is regulated through NF-κB and mitogen-activated protein (MAP) kinase signalling pathways (Ryang *et al.*, 2004). However, *in vivo* studies addressing the pathogenesis of *T. vaginalis* and host responses have for the most part been limited (Cudmmore *et al.*, 2004).

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Pathogen-induced host transcriptional changes in epithelial cells have been described for *Chlamydia trachomatis* (Xia *et al*., 2003), *Helicobacter pylori* (Cox *et al*., 2001), *Pseudomonas aeruginosa* (Ichikawa *et al*., 2000), *Neisseria meningitidis* and *Neisseria gonorrhoeae* (Plant *et al*., 2004) using the micro array technology. Differentially expressed gene profiling using cDNA subtraction has been used as an alternative and complementary tool to microarray analyses, especially in identifying novel genes and transcripts of low abundance (Cao *et al*., 2004). With the objective to identify the transcriptional changes in gene expression during the initial step of *T. vaginalis* adhesion to VECs, we report here our use of the subtraction cDNA library approach. To our knowledge, our data show for the first time that numerous host genes are upregulated upon parasite adherence. We show regulation of select genes using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Further, protein expression analyses and cellular mechanisms of cyclooxygenase 2 (COX-2) expression were studied in more detail. This work has implications for future research directions.

Results

Adhesion of T. vaginalis *T016 to MS-74 VECs*

We optimized an *in vitro* adherence assay between *T. vaginalis* T016 and immortalized MS-74 VECs, as before (Garcia *et al*., 2003). Within 5 min, *T. vaginalis* parasites adhered to MS-74 VECs, and the binding kinetics gave maximum numbers of parasites adhering after 15 min. Figure 1 shows the pictomicrograph of parasites adhered to VECs at 15 min of incubation, and at this time point, the monolayer was intact and parasites had begun to change shape, as reported (Arroyo *et al*., 1993). As significant cytotoxicity and disruption of the monolayer was evident by 45 min, all experiments were carried out using a 30 min time of incubation.

Polymerase chain reaction (PCR)-based subtractive cDNA library

We next used a subtractive cDNA library to study the MA-74 VEC gene regulation induced by *T. vaginalis*. We constructed a cDNA library by subtracting the cDNA of MS-74 bound by *T. vaginalis* parasites (referred to as primed MS-74) from non-primed control MS-74 cDNA. The subtraction methodology involved suppression of polymerase chain reaction (PCR) amplification of the common sequences (Wang and Brown, 1991). The amplified cDNA were ligated to the TA vector to create the library. About 250 clones were obtained in the first round of plating from the transformed cDNA library. The average size of cDNA inserts was ~200 bp. The subtraction efficiency was estimated by comparing the abundance of a known gene before and after subtraction. We amplified the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) from subtracted and unsubtracted cDNA. Figure 2 shows that the GAPDH PCR product is detectable only after ≥25 cycles in the subtracted sample, while it is readily detectable after ≤15 cycles in the unsubtracted sample, indicating successful subtraction efficiency.

MS-74 VEC gene expression regulated by T. vaginalis *adherence*

Sixty rescued plasmid clones were successfully sequenced, of which 85% of the genes were identified and shown in Table 1. Some clones were present in more than one copy. The VEC genes exhibited homologies with the EST or genomic sequences encoding various functional classes of proteins. Further, ~9% were genes of unknown function, and the remaining 6% comprised repeat sequences. About 27% of the upregulated genes fall under the category of cell–cell adhesion, cell structure maintenance and extracellular matrix rearrangement genes, of which fibronectin (FN), plasminogen activator inhibitor (PAI-1) and tetraspan-1 are

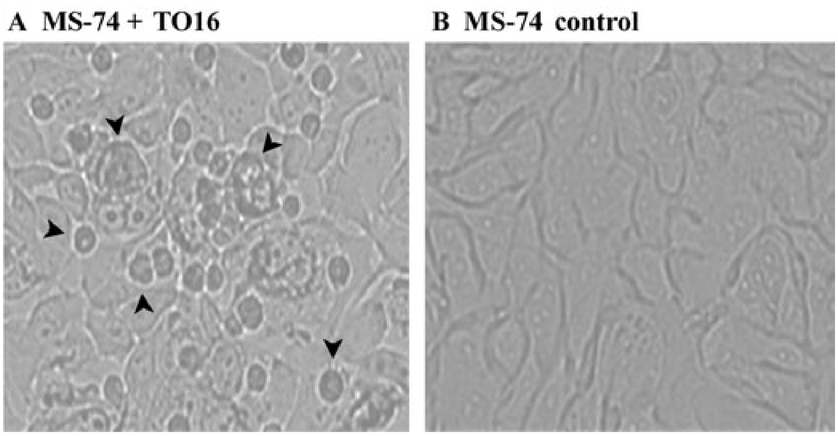

Fig. 1. Adhesion of *T. vaginalis* to immortalized MS-74 vaginal epithelial cells (VECs). MS-74 VEC monolayers in eight-chamber culture slide were incubated with *T. vaginalis* at a parasite to VECs ratio of 5:1. Following 15 min incubation, unbound parasites were washed with PBS. The chambers were removed, and the slide was air dried for observation by microscopy (10× magnification). Trichomonads readily bound to MS-74 VECs (A). Black arrowheads depict some of the bound trichomonads. A control monolayer of MS-74 VECs is shown in (B).

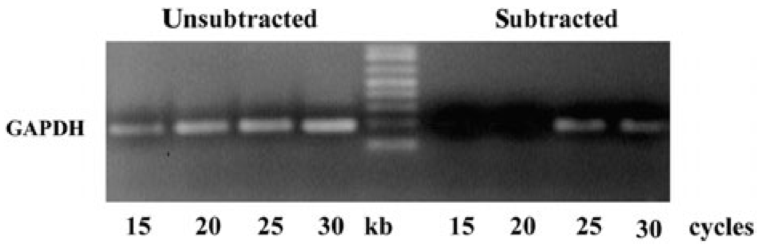

Fig. 2. PCR analysis of subtraction efficiency. PCR was performed on unsubtracted and subtracted cDNAs with GAPDH primers. Aliquots (5 μl) were removed at a predetermined number of cycles and analysed by agarose/ethidium bromide gels. The GAPDH product appeared after 15 cycles in unsubtracted sample but only after 25 cycles in subtracted sample.

predominant. Among cytokine-related genes, monocyte chemotactic protein (MCP-1) and IL-8 were upregulated, suggesting that *T. vaginalis* infection induces proinflammatory responses in the host cells, consistent with the previous reports (Shaio *et al.*, 1995; Ryu *et al.*, 2004). Interestingly, among the upregulated apoptosis-related genes, the prominent ones were the anti-apoptotic members of Bcl2 gene family (defender against apoptotic cell death, DAD1) and COX-2. Two mitochondrial proteins, cytochrome *c* oxidase subunit protein and ATP synthase, were found to be upregulated. Other major upregulated genes include a homologue of cytidine deaminase (APOBEC3C), inflammation and malignancy-related lipocalin, oestrogen receptor-1, and genes involved in protein synthesis.

RT-PCR confirmation of regulation of specific genes

To support the authenticity of the data obtained from the subtraction library, levels of transcripts of specific genes were analysed by quantitative RT-PCR. We selected four genes: (i) the gene for IL-8, a proinflammatory cytokine

Table 1. List of genes upregulated in MS-74 VECs upon early stages of *Trichomonas vaginalis* cytoadherence.

Gene name[a]	Accession No.	Description or function
Cell structure maintenance genes		
CD151	BT020132	Sphingolipid activator protein
Fibronectin 1[5] (FN1)	NM002026	Involved in cell adhesion and morphology
Keratin 14	NM000526	Involved in cytoskeletal rearrangement
KIAA0913	AB020720	Cell-surface protein
Plasminogen activator inhibitor (PAI-1)[3]	AK129790	Serine protease inhibitor
Prosaposin	NM002778	Involved in actin and tubulin folding
Seven transmembrane helix receptor	AB065648	Cell-surface receptor
T-complex polypeptide-1 (TCP-1)	AF026293	Complex forming cell-surface protein
Tetraspan1[3]	NM198902	Transmembrane 4 superfamily gene
Thymosin beta 10	NM021103	Actin-sequestering protein
XB31alpha1	AF438482	Non-classical cadherin
Cytokine genes		
Interleukin 8 (IL-8)	NM000584	Chemotactic and activating cytokine
Monocyte chemotractant protein (MCP-1)	AF519531	Recruitment of monocytes
Apoptosis-related genes		
N-terminal enhancer of split (AES)	NM007005	Induces cell death by forming complexes
Cycloxygenase-2 (COX-2)[5]	AF044206	Inducible form of prostaglandin synthase
Defender against cell death (DAD1)[2]	BC009798	Gene involved in programmed cell death
Dickkopf-3 (Dkk-3) homologue	NM015881	Involved in cell morphological changes
Mitochondrial genes		
ATP synthase[3]	AY714047	Mitochondrial ATP synthase
Cytochrome *c* oxidase[2]	NM144613	Involved in electron transport chain
Others		
Apolipoprotein B mRNA editing catalytic polypeptide (APOBEC3C)[2]	NM021822	Homologue of cytidine deaminase
Oestrogen receptor 1	NM000125	Ligand-activated transcription factor
HLA-B-associated transcript (BAT3)	NM080703	Proline-rich protein
Kallikrein 10	NM145888	Serine protease inhibitor
Lipocalin 2[3]	NM005564	Inflammation and malignancy related
N-deacetylase	NM008306	Involved in deacetylation of heparin
Proactivator polypeptide precursor	NM006287	Precursor protein
Ras homologue	BT019872	Small GTP-binding proteins
Ribosomal protein[2]	BC019014	Protein synthesis
rRNA	AY570524	Protein synthesis
Tissue factor pathway inhibitor (TFPI-2)	NM000602	Serine protease inhibitor
tRNA	AF465979	Protein synthesis

a. Number of copies of the clone picked up out of total 60 clones sequenced.

that has chemotactic and stimulatory effects on T-cells and is known to be stimulated by *T. vaginalis* in monocytes (Shaio *et al*., 1995) and neutrophils (Ryu *et al*., 2004), (ii) the gene for MCP-1, a cytokine capable of inducing T-cell response (Futagami *et al*., 2003), (iii) the gene for COX-2, a highly upregulated proinflammatory molecule known to inhibit apoptosis, promote angiogenesis and tumour invasion (Murata *et al*., 1999) and (iv) the gene for FN, an extracellular matrix glycoprotein implicated in inflammation and angiogenesis (Oyama *et al*., 1991). Fibronectin is known to be a ligand for *T. vaginalis* associations with basement membranes (Crouch and Alderete, 1999). We normalized expression of each gene to GAPDH and compared the expression of primed MS-74 VECs with the non-primed VECs. In addition, we analysed the expression of genes induced by *T. vaginalis*-conditioned medium alone. This was done to determine whether any trichomonad molecules in the conditioned medium induced gene expression in VECs.

RT-PCR products separated on 1% agarose/ethidium bromide gels are shown in Fig. 3A. All four genes had ≥2-fold increased amounts of RT-PCR products in primed MS-74 VECs compared with the controls, confirming the upregulation of genes identified by the subtraction library. Interestingly, except for the MCP-1 gene, the other three genes were induced in the presence of trichomonad conditioned medium alone, suggesting that soluble parasite factors possibly secreted during growth are involved in the upregulation of specific genes. Trypticase-yeast extract-maltose (TYM) alone gave a basal level expression of IL-8, COX-2 and FN1. However, the relative level of expression induced by the conditioned medium is up to threefold higher for these same genes. These data suggest that contact and/or soluble factors of *T. vaginalis* induce host cell responses. Figure 3B presents the levels of increased expression relative to GAPDH as quantified by the scion image beta program, further affirming the net increased expression compared with the controls.

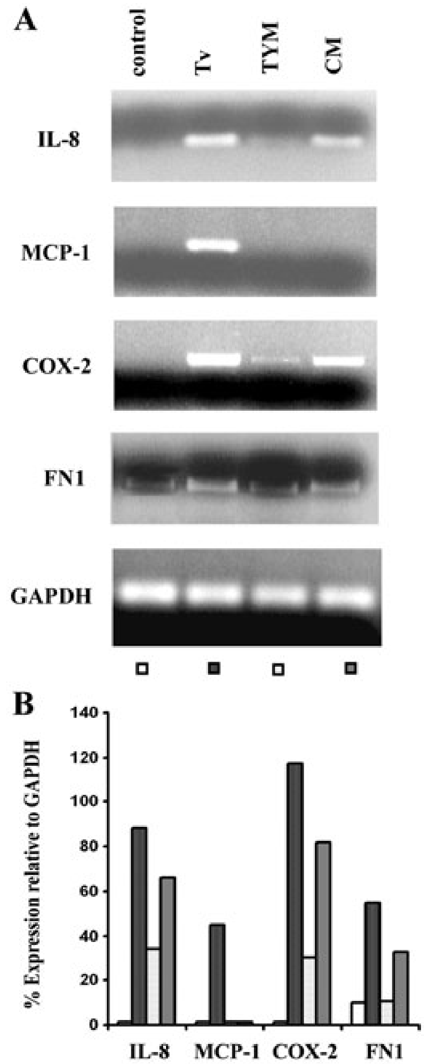

Fig. 3. Confirmation of gene expression patterns in MS-74 VECs by semi-quantitative RT-PCR analyses. For a time period of 30 min, MS-74 VECs were primed with *T. vaginalis* (Tv) and with TYM medium alone or conditioned TYM medium (CM) in which trichomonads were previously grown. These samples were compared with untreated VECs as controls. Total RNA from the primed MS-74 VECs was isolated as detailed in *Experimental procedures* and RT-PCR was performed using gene-specific primers as shown in Table 2.
A. The RT-PCR products separated on 2% agarose ethidium bromide gel.
B. The gene expression pattern relative to GAPDH as a housekeeping control. The values were obtained by scanning the bands from pictures of agarose/ethidium bromide gels using Scion Image beta program, and the per cent expression was relative to baseline GAPDH densities plotted on the graph.

Trichomonas vaginalis *induced COX-2 protein expression in MS-74 VECs*

Cyclooxygenase 2 (COX-2) is an immediate-early response gene induced by growth factors, tumour promoters and oncogenes (Kulkarni *et al*., 2001). We felt that isolation of five COX-2 clones from the 60 selected for analysis was noteworthy. Therefore, we wanted to verify the COX-2 protein expression in primed MS-74 VECs. Figure 4A shows the immunoblot detection of COX-2 protein by anti-COX-2 monoclonal antibody (mAb) in the primed MS-74 cells but not the control VECs. We then performed indirect immunofluorescence studies to detect COX-2 protein localization in the primed MS-74 VECs as seen in Fig. 4B. Fluorescence was readily visualized in the perinuclear region in primed VECs, and all VECs visualized by fluorescence expressed COX-2, consistent with observations made by others (Murakami *et al*., 2003). Hoechst staining shows the alignment of cells in different microscopic fields. These data indicate that the upregulation of COX-2 gene expression is related to increased amounts of protein.

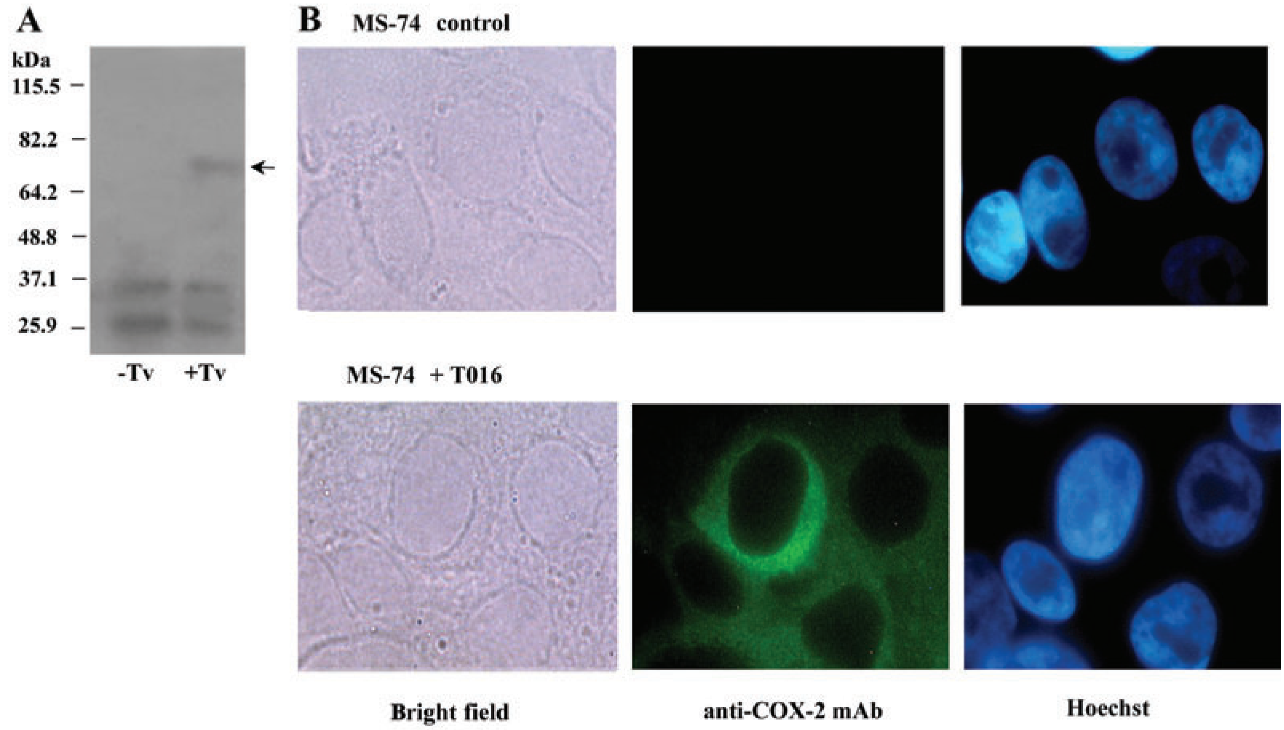

Fig. 4. Detection of COX-2 protein expression in MS-74 VECs in response to *T. vaginalis* adherence.
A. Western blot detection of protein with specific monoclonal antibody (mAb). A confluent monolayer of MS-74 VECs was incubated with trichomonads for 30 min. Cell lysates were prepared from equivalent numbers of primed and non-primed MS-74 VECs, separated by SDS-PAGE and transferred to nitrocellulose membrane for probing with an anti-COX-2 mAb. The molecular weight standards are indicated on the left hand side. The arrow shows the 70 kDa COX-2 protein. A negative control without mAb gave no detection of protein as seen for non-primed VECs.
B. Immunofluorescence detection of COX-2. MS-74 VECs were incubated with *T. vaginalis* parasites. After incubation of VECs with *T. vaginalis* parasites, cells were washed well and incubated with anti-COX-2 mAb followed by FITC-conjugated anti-mouse secondary antibody. The immunostained cells were observed by microscopy (100× magnification). The top panel represents the control non-primed VECs. No fluorescence was detected in the presence of COX-2 antibody. The bottom panel shows the perinuclear expression of COX-2 in VECs incubated with *T. vaginalis* parasites. Hoechst staining shows the alignment of cells.

Kinases are involved in the regulation of COX-2 gene expression

To study the intracellular mechanisms involved in the induction of COX-2 following *T. vaginalis* adherence to MS-74 VECs, we performed experiments inhibiting known protein kinases mediating COX-2 gene transcription (Mahboubi *et al.*, 1998; Korhonen *et al.*, 2004). Before isolation of RNA, VECs first treated with inhibitors were then interacted with parasites. The RT-PCR products of COX-2 were separated on a gel, and the band intensity was quantified using scion image beta program. The relative values are plotted on a graph, as seen in Fig. 5. For calculation purposes, COX-2 expression induced by *T. vaginalis* in the absence of inhibitors was normalized to 100%. The results indicate that SB203580, a p38 MAP kinase inhibitor and Genistein, a tyrosine kinase inhibitor, decreased levels of COX-2 expression by 41% and 31% respectively. In contrast, the p42/44 MAP kinase inhibitor (PD98059) and protein kinase C (PKC) inhibitor (Ro-31-8220) had little or no effect on COX-2 gene expression. These results suggest that p38 MAP kinase and protein tyrosine kinases play an important role in the expression of COX-2 in *T. vaginalis*-stimulated MS-74 VECs. In addition, above results also indicate that PKC is not involved in COX-2 expression in MS-74 VECs.

COX-2 gene expression in response to different trichomonad species

To determine whether COX-2 gene expression was specific to *T. vaginalis*, we tested the related bovine trichomonad *Tritrichomonas foetus* and the non-pathogenic trichomonad *Pentatrichomonas hominis*. As shown in Fig. 6A, *Tri. foetus* also induced COX-2 expression in MS-74 VECs, although to a lesser extent than *T. vaginalis.* To our surprise, the non-pathogenic *P. hominis* showed poor, if any, induction of COX-2 gene expression (20%), when compared with that of *T. vaginalis* and *Tri. foetus* (65%) (Fig. 6B).

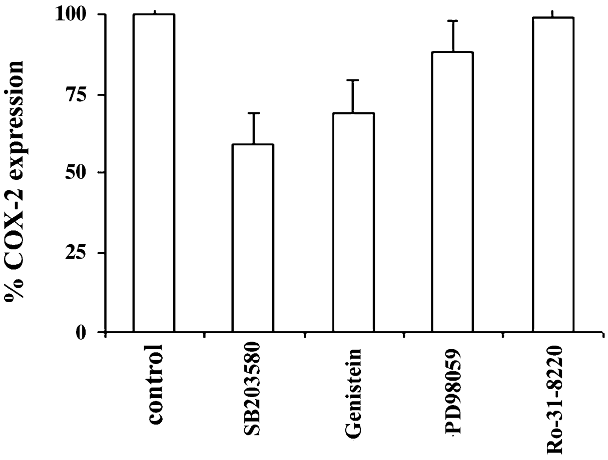

Fig. 5. Regulation of COX-2 expression in MS-74 VECs in the presence of pharmacological compounds. VECs were cultured as described in *Experimental procedures* and were exposed to different pharmacological compounds or without additions as a negative control, as indicated. VECs were then incubated with *T. vaginalis* for 30 min after which VECs were harvested for total RNA isolation. COX-2 expression was determined by RT-PCR. The PCR products were separated on 1% agarose/ethidium bromide gel. The bands were quantified using scion image beta program. The values were plotted on the graph. Expression in the absence of any compound was considered as 100% for comparison purposes. Identical results were obtained from three independent experiments performed identically.

Discussion

In this study we used the suppression subtractive hybridization technique to evaluate any responses of the VECs to *T. vaginalis* adherence. We have identified several genes that are upregulated upon initial parasite adherence to host cells, which may have implications for understanding the early network of host responses operating in *T. vaginalis* infections. A high efficiency of subtraction was evidenced by the GAPDH housekeeping gene amplification (Fig. 2). We appreciate and were not surprised that a small fraction of GAPDH message was still retained, as can be the case for this subtraction procedure (Buchaille *et al*., 2000). From 60 cDNA clones we identified a set of genes with increased levels of transcription in response to contact with *T. vaginalis* (Table 1).

Interestingly, ~27% of the genes were associated with cell structure maintenance and extracellular matrix (ECM) rearrangement, of which the major ones were FN and PAI-1. These data suggest that trichomonads may regulate cell interactions with ECM by co-ordinated induction of FN and PAI-1 (Liu *et al*., 2000) and result in a robust signal transduction process. Furthermore, *T. vaginalis* attaches to the basement membrane components FN and laminin in a receptor-mediated fashion (Crouch and Alderete, 1999). *T. vaginalis* also contains two fibronectin-like binding proteins, flp1 and flp2, that are regulated by iron (Crouch and Alderete, 2001). It is conceivable that trichomonads use FN-mediated binding after expression of FN by VECs as an alternative way of colonizing the vaginal epithelium. This mechanism would be in addition to the four well-characterized trichomonad adhesins (Arroyo *et al*., 1992; Garcia *et al*., 2003; Mundodi *et al*., 2004). In this study, we have shown that two major proinflammatory cytokines IL-8 and MCP-1 are upregulated upon parasite adherence to VECs. Consistent with our finding, we know that trichomonosis is characterized by severe inflammation and tissue cytotoxicity (Krieger, 1981) and that IL-8 is present in vaginal discharges from patients (Shaio *et al*., 1994; 1995). Also, recent reports show that human neutrophils and monocytes stimulated by *T. vaginalis* are induced to produce IL-8 (Shaio *et al*., 1995; Ryu *et al*., 2004). Similarly, IL-8 is also induced during the early stages of infection by *Candida albicans* (Nomanbhoy *et al*., 2002) and *N. gonorrhoeae* (Fichorova *et al*., 2001). MCP-1 is considered critical for migration of cells to sites of infection, and the expression levels are elevated in the

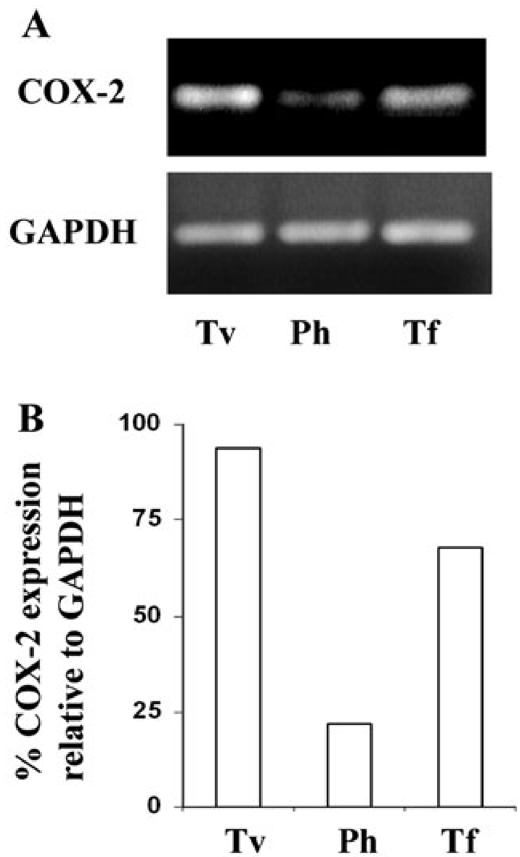

Fig. 6. Expression of COX-2 in MS-74 VECs in response to different trichomonad species. VECs were incubated with *Trichomonas vaginalis* (Tv), *Pentatrichomonas hominis* (Ph) or *Tritrichomonas foetus* (Tf) for 30 min. After washing for removal of the unbound trichomonads, VECs were used for RNA isolation. RT-PCR was performed on total RNA using COX-2 and GAPDH primers. The PCR products separated on 1% agarose gel and stained by ethidium bromide (A). The per cent expression of COX-2 relative to GAPDH is represented in (B) in a bar graph.

vagina of mice during *C. albicans* infection (Fidel *et al*., 1999) and in human VECs infected with *C. albicans* (Nomanbhoy *et al*., 2002). MCP-1 is also upregulated in epithelial cells stimulated with other pathogens, such as *H. pylori* (Futagami *et al*., 2003) and *Yersinia enterocolitica* (Kampik *et al*., 2000).

Host cells often respond to pathogens by undergoing apoptosis as a self-defence mechanism to remove infected cells. The major upregulated apoptosis-related genes in our VEC subtraction library were DAD1, a member of Bcl2 gene family (Cory *et al*., 2003) and COX-2. These genes are anti-apoptotic in nature, suggesting that the trichomonads might resist the host cell apoptosis because of its self-limiting nature of infection. COX-2 is known to be induced in several cell types by bacterial lipopolysaccharide (LPS) (Yang *et al*., 1999; Dieter *et al*., 2002) and in presence of secreted proteins from *N. meningitidis* (Robinson *et al*., 2004). It is possible that *T. vaginalis* organisms possess some soluble factor to induce COX-2 gene expression (Fig. 3A). Alternatively, COX-2 may be induced by the upregulation of cytokines and other growth factors that are increased upon *T. vaginalis* adherence.

Other genes from the subtraction library include the mitochondrial-associated genes ATP synthase and cytochrome *c* oxidase subunit protein, indicating that mitochondrial dysfunction may be induced by *T. vaginalis* cytoadherence. This is also seen in *Streptococcus pyogenes*-infected epithelial cells (Nakagawa *et al*., 2004). Other major upregulated genes include apolipoprotein B mRNA editing polypeptide (APOBEC3C), lipocalin 2 and oestrogen receptor 1(ESR). Importantly, APOBEC3C is known to exhibit potent DNA mutator activity in an *Escherichia coli* assay (Harris *et al*., 2002). ESR is a transcription factor that is activated in the presence of oestrogen, which is known to play a role in the progression of breast cancer in humans (Wang *et al*., 1997).

We confirmed the expression of selected genes using semi-quantitative RT-PCR and tested whether upregulation at the transcriptional level reflected increased protein expression. IL-8, MCP-1, COX-2 and FN were selected for RT-PCR analysis, and highly upregulated expression of the four genes was seen (Fig. 3). Interestingly, the soluble factors secreted into the conditioned medium also induced upregulation of IL-8, COX-2 and FN. That MCP-1 was not induced under this condition suggests specificity in that trichomonad contact with VECs is prerequisite for the induction. The relative COX-2 gene expression value was the highest when compared with the rest of the genes, which reaffirmed the relative abundance of COX-2 clones in the subtraction library. That COX-2 protein was indeed induced by MS-74 VECs upon *T. vaginalis* adherence (Fig. 4) was further confirmed by the possible role of p38 MAP kinase inhibitor and to a lesser extent by tyrosine kinase inhibitor (Fig. 5). It has been shown that p38 MAP kinase inhibition leads to decreased COX-2 expression in *Lactobacillus rhamnosus*-stimulated epithelial cells (Korhonen *et al*., 2004) and in LPS-treated human monocytes (Dean *et al*., 1999). Interestingly in a recent study, p38 MAP kinase was implicated in *T. vaginalis*-induced activation of human macrophages (Ryang *et al*., 2004).

The response to the bovine trichomonad *Tri. foetus* in the reproductive tract is similar to the response to *T. vaginalis* (Corbeil *et al*., 1998). We found significant but unequal upregulation of COX-2 expression in VECs stimulated by *T. vaginalis* and *Tri. foetus* (Fig. 6). We believe the lower extent of COX-2 increase by *Tri. foetus* may be attributable to the lower levels of adherence to human VECs (Singh *et al*., 2004). In contrast, the non-pathogenic *P. hominis* showed very low upregulation of COX-2, suggesting that COX-2 induction is important for the pathogenesis of *T. vaginalis*. The result using *P. hominis* may reflect the specific tropism for the intestinal tract and the absence of adherence to VECs by this parasite.

Several studies have linked *T. vaginalis* pathogenesis with cervical cancer (Yap *et al*., 1995; Zhang *et al.,* 1995; Sayed el-Ahl *et al*., 2002). COX-2 is an inducible form of the enzyme involved in the production of prostanoids and is often upregulated in many inflammatory diseases. Enhanced synthesis of prostaglandins induced by COX-2 stimulates cancer cell proliferation (Tsuji *et al*., 2001), inhibits apoptosis and promotes angiogenesis (Tsujii *et al*., 1998; Gupta *et al*., 2003) in various cell types. COX-2 is consistently overexpressed in all primary and metastatic epithelial cancers, including prostate (Gupta *et al*., 2000) and cervical cancers (Kulkarni *et al*., 2001). COX-2 is upregulated during the pathogenesis of *H. pylori* infections (Akhtar *et al*., 2001), which is in turn linked to gastric cancer. It is also noteworthy that tobacco smoking results in increased upregulation of FN, which in turn is responsible for lung cell carcinoma progression. Moreover, upregulation of FN is associated with increased amounts of COX-2 mRNA and protein (Han *et al*., 2004). A similar scenario might be envisioned during trichomonosis.

Experimental procedures

Parasites and host cells

Trichomonas vaginalis isolate T016 was grown in TYM medium supplemented with 10% heat-inactivated donor horse serum at 37°C (Diamond, 1957). Immortalized MS-74 human VECs (Klumpp *et al*., 2002) were used for adherence experiments and were grown in Dulbecco's modified Eagle medium (D-MEM) (Invitrogen) supplemented with 10% fetal bovine serum, at 37°C, in the presence of 5% CO_2.

Adherence assay and isolation of total RNA

The MS-74 VECs were used for adherence as recently detailed (Garcia *et al*., 2003). Briefly, 6×10^5 VECs were seeded onto T75 culture flasks and allowed to form a monolayer for 2 days. VECs were then washed with a medium mixture of D-MEM:TYM (2:1, v/v) without serum. Parasites at mid-logarithmic phase of growth were added to the MS-74 monolayer at a parasite:VEC ratio of 10:1 and incubated at 37°C to allow for parasite adherence. Unbound and loosely adhered parasites were washed, and the monolayer was incubated on ice for an additional 1 h. The monolayer was washed thoroughly with ice-cold PBS to remove all bound parasites until no parasites were detected by microscopy (Alderete and Garza, 1985). The monolayer of primed VECs were collected, washed with PBS and used for RNA isolation. Total RNA from control non-primed VECs and primed VECs after contact with *T. vaginalis* was isolated using Trizol reagent (Invitrogen).

For isolation of RNA from MS-74 VECs treated with different kinase inhibitors (Sigma Aldrich), the monolayer was grown to confluency in individual wells of 24-well culture plates. Before incubation with *T. vaginalis*, the monolayer was incubated for 4 h with SB203580 (1 μmol l^{-1}, an inhibitor of p38 MAP kinase), genistein (100 μmol l^{-1}, an inhibitor of protein tyrosine kinase), PD98095 (10 μmol l^{-1}, an inhibitor of p42/44 MAP kinase) and Ro 31-8220 (1 μmol l^{-1}, an inhibitor of protein kinase C). Trichomonads were then added, and after 30 min of incubation VECs were used for RNA isolation.

Synthesis of cDNA and construction of subtractive cDNA library

cDNA from primed MS-74 VECs (tester cDNA) and non-primed VECs (driver cDNA) were prepared using a cDNA synthesis system (Roche Diagnostics). Briefly, 1 μg of total RNA was reverse transcribed using an oligo $(dT)_{15}$ primer and AMV reverse transcriptase according to manufacturer's protocol. The subtractive cDNA library was constructed according to the standard protocol (Wang and Brown, 1991). Briefly, each set of cDNA was digested with *Alu*I and *Rsa*I, to generate shorter, blunt-ended cDNA fragments. The tester DNA and driver DNA were ligated with different sets of adaptors. Ligated cDNA was amplified by PCR using the adaptor primers to obtain large amounts of cDNA. The tester cDNA was labelled with [^{32}P]-dCTP and the driver cDNA was biotinylated using 0.5 mM bio-11-dUTP (Biotium). A first hybridization between an excess of driver and tester was carried out to enrich the differentially expressed sequences. Tracer/driver and driver/driver hybrids and biotinylated single-stranded driver cDNA were removed by addition of streptavidin and extraction with phenol/chloroform. The per cent of tracer cDNA removed was determined by the radioactive counts remaining after phenol/chloroform extraction. Further rounds of subtraction were performed using the cDNAs from the previous round as template for PCR synthesis of tracer and driver cDNAs. After 10 rounds of alternating long and short hybridizations, the subtracted cDNA fraction was cloned into a TA vector and transformed into *E. coli* to create a primed MS-74 cDNA library.

Polymerase chain reaction analysis of the subtraction efficiency

Polymerase chain reaction was performed on subtracted and unsubtracted cDNA with the housekeeping gene GAPDH primers (Table 2). Each reaction was amplified for 30 cycles and 5 μl aliquots were removed after 15, 20 and 25 cycles. The PCR products were electrophoresed on a 1% agarose/ethidium bromide gel. The efficiency of subtraction was reflected by the difference in the number of cycles required for equal amplification of the PCR product in subtracted and unsubtracted samples.

Sequencing and analysis

Colonies were randomly selected and plasmids were prepared using a miniprep kit (Qiagen). cDNA inserts were verified by restriction digestion, and the clones were sequenced in our institutional DNA sequencing facility. Sequence data were compared with data in GenBank using a BLAST program.

RT-PCR analysis of selected genes

Total RNA from non-primed VECs, primed VECs, and VECs incubated with TYM alone or *T. vaginalis*-conditioned medium was isolated using Trizol reagent. Total RNA (1 μg) was reverse transcribed with oligo (dT) primer using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. PCR amplification of cDNA was carried out using gene-specific primers (Table 2). GAPDH was used as an internal control. PCR products were separated on 2% agarose/ethidium bromide gels, and the band intensity was quantified using the Scion image beta program.

SDS-PAGE and Western blotting

Equal amounts of lysate from primed and non-primed VECs were separated on 10% SDS-PAGE, and the proteins were transferred onto nitrocellulose membranes (Bio-Rad). The nitrocellulose

Table 2. List of primers used for RT-PCR analyses.

Gene name	Primer sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)
GAPDH	Sense CCATGGAGAAGGCTGGGG Antisense CAAAGTTGTCATGGATGACC	60	200
IL-8	Sense ATGACTTCCAAGCTGGCCGTGGCT Antisense TCTCAGCCCTCTTCAAAAACTTCTC	56	265
MCP-1	Sense TCCTGTGCCTGCTGCTCATAG Antisense TTCTGAACCCACTTCTGCTTGG	57	565
COX-2	Sense CAGCACTTCACGCATCAGTT Antisense TCTGGTCAATGGAAGCCTGT	60	756
FN1	Sense TGGATCCATGAACTTTCTGCTGTC Antisense TCACCGCCTTGGCTTGTCACAT	48	250

blots were blocked in 0.1% Tween 20 and 5% BSA and subsequently probed with anti-COX-2 mAb (Cayman chemicals). The blot was further incubated with anti-mouse secondary antibody conjugated with horseradish peroxidase (Bio-Rad). The blot was washed well and incubated in horseradish peroxidase substrate (Bio-Rad) to visualize the reactive band.

Immunofluorescent staining

MS-74 VECs were seeded on Falcon 8 chamber culture slides (Becton Dickinson) and allowed for monolayer formation. Trichomonads were added to the monolayer at a parasite to cell ratio of 10:1 and incubated for 30 min at 37°C. The monolayer was then washed to remove free and unbound trichomonads. The monolayer on the slide was incubated in blocking buffer of PBS containing 5% BSA followed by the addition of 1:1000 dilution of COX-2 mAb for 1 h. Fluoresceine isothiocyante-conjugated anti-mouse antibody was then added for 30 min at 4°C. The monolayer was washed with PBS and incubated with Hoechst stain (0.1 mg ml^{-1}) for 10 min and again washed with PBS. Finally, the chambers on the slide were removed, and slides were processed for observation using an Olympus BX41 microscope.

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