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Effects of bacterial products on enterocyte–macrophage interactions *in vitro*

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ABSTRACT

We describe a coculture model of a human intestinal epithelial cell line and human peripheral blood monocytes in which monocytes differentiate into cells with features of resident intestinal macrophages. Caco-2 cells are grown on the lower surface of a semipermeable filter with pore size of 3 μm (Transwells®) until they differentiate into enterocytes. Peripheral-blood monocytes are added and the co-culture incubated for two days. Monocytes migrate through the pores of the membrane, come into direct contact with the basolateral surfaces of the epithelial cell monolayer, and develop characteristics of resident intestinal macrophages including downregulation of CD14 expression and reduced pro-inflammatory cytokine responses (IL-8, TNF and IL-1β) to bacterial products. The apical application of lipopolysaccharide (LPS) and muramyl dipeptide (MDP) resulted in an increased number of integrated monocytes, but abrogated the downregulation of CD14 expression and the diminished cytokine responses. MDP also reduced tight-junctional integrity, whilst LPS had no effect. These data indicate that LPS and MDP have significant pathophysiological effects on enterocyte–monocyte interactions, and confirm other studies that demonstrate that enterocytes and their products influence monocyte differentiation. This model may be useful in providing insights into the interaction between monocytes, epithelial cells and intestinal bacteria in health and disease.

1. Introduction

The epithelium of the human intestinal tract lies at the interface between the host immune system and a vast array of potentially pathogenic microorganisms. There are a number of mechanisms that provide a potent physical barrier against invasive bacteria, including the presence of tight-junctions between enterocytes and an extensive glycocalyx that serves as a diffusion barrier [1].

The main cellular component of the intestinal innate immune system is the resident or tissue macrophage that lies immediately beneath the epithelial cell basement membrane. These cells make up 10–20% of the lamina propria cell number and are the largest pool of mononuclear phagocyte-derived cells in the body [2].

Human resident intestinal macrophages possess avid phagocytic and bactericidal capabilities, but do not express pathogen recognition receptors or other receptors associated with the innate immune response. These include the receptors for lipopolysaccharide (LPS) (CD14), complement receptor 3 (CR3) (CD11b/CD18), and CR4 (CD11c/CD18) and the toll-like receptors (TLR), TLR2 and TLR4 [3–6]. Nor do they produce proinflammatory cytokines, such as IL-1β, in response to an array of inflammatory stimuli such as LPS, and interferon (IFN)-γ. This process of down-regulation is mediated by transforming growth factor beta (TGF-β) produced by nearby stromal mast cells and epithelial cells [7]. The phenotype of the intestinal macrophage has functional implications, many of which are readily apparent, e.g. LPS-unresponsiveness due to down-regulation of CD14 and TLR4.

During acute inflammation, there is an influx of CD14-positive blood monocytes [3]. Unlike resident macrophages, these recruited cells respond to LPS and activating signals by producing increased amounts of pro-inflammatory cytokines, reactive oxygen and nitrogen intermediates, a variety of proteases that degrade the extracellular matrix and other potentially tissue-damaging molecules (reviewed in Mahida YR [8]). Epithelial cell barrier function is also affected [9], a process mediated in part by monocyte-derived TNF-α [10] and IL1-β [11]. In uncontrolled inflammatory responses, tissue damage is likely to be mediated by these monocyte- and T-cell-derived molecules. The effectiveness of neutralising monoclonal antibodies in the clinic (e.g. anti-TNF-α antibodies, such as infliximab [12] and in experimental models of inflammation (e.g. anti-IFN-γ) in preventing intestinal damage supports this assumption.

A number of investigators have sought to develop *in vitro* models to study the interaction between enterocytes and macrophages.

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It is possible to examine resected intestinal specimens in Ussing chambers, but isolated tissues only survive for about 2 h, greatly restricting the scope of studies that can be performed. A number of *in vitro* culture models used enterocyte cell lines separated from monocytes by a semi-permeable Transwell® membrane [10,13,14]. However, such models may not allow cell-cell contact to occur reliably: monocytes are added to the lower, basolateral chamber of the co-cultures beneath the inserts, or the size of the pores (0.4 μm) within the membrane of the Transwells® are too small to allow monocytes to migrate to the epithelial cell layer [10,13,14] also used Transwells®, but of 3 μm pore diameter, separating HT-29 epithelial cells and monocytes, but did not observe the differentiation into a phenotype typical of intestinal macrophages, presumably because of the absence of cell–cell interactions.

To address these issues, we created an *in vitro* culture system that allows contact to take place between enterocytes and monocytes. This system is based on the *in vitro* M-cell models developed by Kerneis et al. [15] and modified by Tyrer et al. [16], and a model of enterocyte/dendritic cell interactions by Rescigno et al. [17]. In this model, freshly isolated human monocytes are added to Caco-2 cells cultured on the lower surface of Transwell® inserts. Monocytes integrate into the epithelial monolayer and the expression of macrophage markers and secretion of cytokines are consistent with those seen in intestinal macrophages. We used this technique to investigate epithelial-monocyte responses to the inflammatory bacterial pathogen-associated molecular pattern molecules (PAMPs), LPS and muramyl dipeptide (MDP) and found that they had significant pathophysiological effects in these co-cultures.

2. Materials and methods

2.1. Materials

The human colon adenocarcinoma cell line Caco-2 was obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. 75 cm^2 tissue culture flasks were purchased from Sarstedt, Australia. 3.0 μm diameter pore, polycarbonate Transwells were purchased from Corning Costar. Ficoll-Paque was bought from GE Healthcare. Invitrogen supplied Dulbecco's Minimal Eagle Medium (DMEM) with Glutamax I, 4.5 g/l glucose and pyridoxal, foetal calf serum (FCS), minimal Eagle medium (MEM) non-essential amino acids, Antibiotic/Antimycotic™, RPMI 1640, Dulbecco's phosphate buffered saline (D-PBS), Alexa-546 conjugates, chloromethylfluorescein diacetate (CMFDA), and ProLong Antifade Kit. D-PBS was also obtained from Amresco, Solon, Ohio, USA. The EVOM epithelial voltage meter was purchased from World Precision Instruments, USA. Primary antibodies and IL-8, TNF and IL-1β enzyme-linked immunosorbent assay kits were purchased from BD Pharmingen. RosetteSep Monocyte Isolation Kit was bought from Stem Cell Technologies. LPS (rough strains) from *Salmonella enterica* serotype Minnesota Re 595 (Re mutant) and synthetic MDP were bought from Sigma. TGF-β ELISA kit was sourced from R&D Biosciences. Diff-Quik came from Dade-Behring (Switzerland).

2.2. Experimental procedures

2.2.1. Caco-2 cell cultures

Caco-2 cells were cultured as described in Tyrer et al. [13] and shown schematically in Fig. 1. Prior to co-culture, the transepithelial electrical resistance of the Caco-2 cultures was measured with an EVOM (World Precision Instruments, USA) to confirm their fully differentiated state. Transepithelial electrical resistance (TEER) is a measure of tight junction integrity and indicator of differentiation. Cultures displaying a TEER greater than 150 Ω cm^2 were considered to have differentiated into enterocyte-like cells and suitable for co-culture.

2.2.2. Monocyte isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood with Ficoll-Paque according to manufacturers' instructions. The PBMCs were resuspended in 2 %v/v heat-inactivated FCS in D-PBS. To this solution, 30 μl 100 mM EDTA and 150 μl RosetteSep were added and the sample left on ice for 15 min. 300 μl of diluted red blood cells (1 ml red blood cells and 4 ml D-PBS) was added and the mixture left on ice for 10 min. An equal volume of 2%v/v FBS, 1 mM EDTA in D-PBS was added and underlaid with 4 ml Ficoll. The sample was spun for 20 min, 912*g* at 20 °C with no braking. The interface was removed and transferred to a new 15 ml centrifuge tube and the monocytes were washed twice in 10 ml of 2 %v/v FBS in D-PBS (659*g* for 10 min at 20 °C with braking). The monocytes were then resuspended in 3 ml 2 %v/v FBS in PBS and counted by Trypan blue exclusion assay. For purity, monocytes were checked by DiffQuik assay and were found to be more than 95% enriched.

2.2.3. Caco-2-monocyte co-culture

The isolated monocytes were labelled with a fluorescent dye to facilitate their identification in co-cultures. The monocytes were incubated for 45 min at 37 °C, 5% CO_2 in the presence of 5 μM CMFDA, washed three times and resuspended to 4×10^6 cells/ml in Caco-2 culture medium. The basolateral media from the Transwells® was replaced with 100 μl of the cell suspension. 100 μl of Caco-2 media was used in control Transwells. The apical media were removed and replaced with either 0.6 ml Caco-2 media (control), 100 ng/ml LPS in Caco-2 media or 100 μg/ml MDP in Caco-2 media. The doses were chosen to reflect physiological concentrations of these bacterial products in the intestinal lumen [18]. All cultures were incubated for 2 days at 37 °C, 5% CO_2.

2.2.4. Immunofluorescence studies

Transwells were washed three times in D-PBS and fixed in ice-cold ethanol. The samples were blocked with 2 %w/v bovine serum albumin in D-PBS for 1 h at 37 °C. The Transwells were incubated at 37 °C for hour in the presence of primary antibody, before washing three times in D-PBS. The samples were probed with 1/500 dilution of appropriate Alexa-546 conjugate for 1 h at 37 °C, washed and counterstained for 15 min at room temperature with 1 μg/ml DAPI. After further washing, the Transwell membranes were excised by use of a scalpel and placed onto glass slides. Coverslips were mounted with ProLong Antifade Kit.

2.2.5. Fluorescence microphotography and image analysis

Slides were photographed with a Nikon TE300 microscope and Princeton Instruments MicroMax cooled CCD camera. Single cell total fluorescence intensities were used to determine the level of marker staining and hence protein expression in the cultures, the number of CMFDA labelled monocytes that integrated into the monolayers, and the numbers of condensed nuclei within the epithelial monolayers were determined with MetaMorph. The data from image analysis was obtained from five fields of view from five samples, with a ×20 objective.

For *x–y* orthogonal reconstruction, cultures were imaged at 1 μm intervals, and the consequent image stacks deconvolved with a nearest-neighbour algorithm with MetaMorph. *X–y* and rocking projection were created with NIH ImageJ.

2.2.6. Cytokine analysis

Media was withdrawn from the basolateral aspect of the cultures and frozen at −20 °C until analysis for IL-8, TNF, IL-1β and TGF-β by ELISA. Experiments were performed in triplicate. For experiments performed on monocytes only, monocytes were isolated as described above and 100 μl of cell suspension plated into 96-well microtitre plates in RPMI 1640 plus 2 %v/v FCS at a concen-