Toxicology in Vitro 24 (2010) 1441–1449

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation

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ARTICLE INFO

Article history:
Received 24 November 2009
Accepted 12 April 2010
Available online 18 April 2010

Keywords:
Inflammation
Caco-2
IBD modeling
Cytokine
Lipopolysaccharide
Costimulation
Differentiation

ABSTRACT

Enterocytes regulate gut maintenance and defence by secreting and responding to inflammatory mediators and by modulating the intestinal epithelial permeability. In order to develop an *in vitro* model of the acute phase of intestinal inflammation, Caco-2 cells were exposed to the inflammatory mediators IL-1β, TNF-α, IFN-γ and LPS, and the importance of several experimental parameters, i.e. cell differentiation, stimulus nature, concentration and combination on the inflammatory response was assessed by measuring the production of IL-6, IL-8, PGE-2 and NO and by evaluating the monolayer permeability. A maximal increase in IL-8, IL-6 and PGE-2 production and monolayer permeability was observed when using the cytokines simultaneously at their highest level, but this relied mainly on IL-1β. The effects of TNF-α on IL-8 and IL-6 or NO production were stronger upon combination with IL-1β or IFN-γ, respectively, whereas cells were unaffected by the presence of LPS. Although NO production, induced by IFN-γ-containing combinations, was observed only in differentiated cells, general inflammatory response was higher in proliferating cells. The use of a mixture of IL-1β, TNF-α and IFN-γ thus accurately mimics intestinal inflammatory processes, but cell differentiation and stimuli combination are important parameters to take into account for *in vitro* studies on intestinal inflammation.

1. Introduction

Intestinal inflammation is a natural and protective process, which is crucial to maintain gut integrity and functioning (Martin and Wallace, 2006). It requires a continuous crosstalk between the different cell types present in the gut, and results from and in the secretion of soluble mediators such as cytokines, eicosanoids, nitric oxide (NO) and growth factors (Böcker et al., 2003; Martin and Wallace, 2006; Danese et al., 2008). In turn, these mediators stimulate or attenuate the secretion of other mediators by the target cells, and their expression thus has to be finely regulated to obtain a coordinated host's response.

Intestinal epithelial cells (IECs) have a strategic position at the interface between the antigenic luminal environment and the internal milieu. They actively contribute to the gut immune system, mediating processes as mucosal defense, tolerance to resident flora and barrier repair (Cario et al., 2000). IECs establish bidirectional interactions with the underlying immune cells, and participate to the mucosal inflammatory response in two ways (Böcker et al., 2003; Danese et al., 2008). Their property of selectively modulating the permeability of the epithelial monolayer – and thus immune cell exposure to antigens – as well as their ability to synthesize and secrete inflammatory mediators themselves, allow them to trigger immune cells and initiate the inflammatory process. Conversely, IECs also respond to various inflammatory mediators secreted by the immune cells, by modulating the epithelial monolayer permeability and secretion, thus further amplifying or attenuating the inflammatory process (Jung et al., 1995; Panja et al., 1998; Bruewer et al., 2005).

Although intestinal inflammation is a continuous and protective process, a dysregulation of one of its components can lead to severe intestinal disorders. Inflammatory bowel diseases (IBDs), the collective name for Crohn's disease and ulcerative colitis, are characterized by chronic and unpredictable attacks of inflammation of the intestine, causing weight loss, diarrhoea, rectal bleeding, abdominal pain, fever and anemia (Neuman, 2007). IBDs affect about 0.5–1% of the population in Western countries and incidence levels are increasing worldwide (Russel, 2000). The pathogenesis of IBDs is not fully elucidated, but two central features associated with IBDs, i.e. defective epithelial cell barrier functioning and an exaggerated immune activity, are thought to cooperate in a self-amplifying loop, where barrier dysfunction causes increased paracellular permeation of harmful luminal antigens and thus

Abbreviations: COX, cyclo-oxygenase; IBDs, inflammatory bowel diseases; IECs, intestinal epithelial cells; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; TEER, transepithelial electrical resistance.

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0887-2333/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.tiv.2010.04.002

increases activation of mucosal immune cells, leading in turn to increased release of inflammatory stimuli, IEC response and further barrier dysfunction (Bruewer et al., 2003; Wang et al., 2006; Al-Sadi and Ma, 2007).

Several inflammatory mediators are thought to be implicated in the development of IBDs. However, *in vitro* research on IECs has mainly focused on the involvement of three major cytokines, i.e. Interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ. IL-1β is a multifunctional cytokine playing a major role both in the initiation and the amplification of many inflammatory conditions (Böcker et al., 1998). It is released by various cell types including monocytes–macrophages, neutrophils and endothelial cells (Martin and Wallace, 2006) and has been found in increased concentrations in the intestinal tissue of IBD patients (Ligumsky et al., 1990; Reinecker et al., 1993; Reimund et al., 1996). IL-1β mediates important features of IBD, such as the generation of fever, the reduction of appetite, the release of mediators and the recruitment of leukocytes (Martin and Wallace, 2006). TNF-α and IFN-γ are cytokines that play a key role in the acute phase of inflammation and diverse immunological processes, modulating e.g. the recruitment and adhesion of leukocytes and the antigen presentation (Rosenstiel et al., 2003; Schlottmann et al., 2004). Their expression levels have been found elevated in the mucosa of IBD patients (Fais et al., 1991; Reimund et al., 1996), notably due to an increased secretion by Th-1 lymphocytes of the *lamina propria* and, in case of TNF-α, by the IECs themselves.

The presence of bacterial components, such as lipopolysaccharides (LPS), is also considered as an important factor both in the initiation and in the reactivation of IBDs: a dysregulation of IEC sensitivity to the common local microflora is believed to cause an initial inappropriate inflammatory stimulus, leading to an exaggerated cytokine presence and IBD development (Caradonna et al., 2000; Cario et al., 2000; Böcker et al., 2003). Moreover, the leaky IEC barrier observed during IBDs leads to increased permeation to bacteria during inflammation, which further enhances immune activity (Aoki, 1978; Bruewer et al., 2005).

The *in vitro* effects of IL-1β, TNF-α, IFN-γ and LPS on IECs consist in activation of intracellular cascades, leading to an increased transcriptional activity and the secretion of interleukin (IL)-8 (Schuerer-Maly et al., 1994), IL-6 (Parikh et al., 1997; Ogle et al., 1997; Vitkus et al., 1998), prostaglandin (PG)-E2 (Grishin et al., 2004; Wright et al., 2004; Duque et al., 2006) and/or NO (Chavez et al., 1999; Forsythe et al., 2002), as well as increase of the paracellular permeability through defects in tight junction functioning or assembly (Forsythe et al., 2002; Ma et al., 2005; Bruewer et al., 2005; Al-Sadi and Ma, 2007).

These studies however show a lack of homogeneity in experimental conditions such as stimulus concentration, exposure duration, and nature and differentiation stage of cells. Moreover, although IL-1β, TNF-α, IFN-γ and LPS are individually known to contribute to the relapse of inflammation, reports on their concerted action as occurring during the acute phase of IBDs are scarce (Ou et al., 2009). In view to establish a valid and consistent *in vitro* model of the physio-pathological behaviour of enterocytes during the acute phase of IBDs, we exposed Caco-2 cells, a well-established and widely used model of the human intestinal barrier, to IL-1β, TNF-α, IFN-γ and LPS exposure, and evaluated inflammatory parameters with respect to cell differentiation, stimulus concentration and stimuli combination.

2. Materials and methods

2.1. Chemicals

Culture reagents were from Lonza (Verviers, BE) unless mentioned otherwise. IL-1β, TNF-α, and LPS were purchased from Sigma–Aldrich (St. Louis, MO) and IFN-γ was from Calbiochem (Darmstadt, DE). Arachidonic acid, taurocholate and Triton-X-100 were from Sigma.

2.2. Cell culture

The Caco-2 cell line, derived from a human colon adenocarcinoma (ATCC, Rockville, MD), was used between passages 30 and 50 and cultured in DMEM containing 4.5 g/l glucose, 25 mM hepes, 10% (v/v) heat-inactivated FBS (Hyclone Perbio-Sciences, Erembodegem, BE), 2% (v/v) L-glutamine 200 mM and 1% (v/v) nonessential amino acids (NEAA) (Invitrogen, Carlsbad, CA). Cells were grown on 175 cm^2 flasks (Greiner Bio-One, Strickenhausen, DE) in an atmosphere of 5% CO_2/95% air (v:v) at 37 °C. For experiments, cells were seeded at a density of 40×10^3 cells/cm^2 on type I collagen (Sigma–Aldrich) precoated 24-well plates (Nunc, Roskilde, DK) and cultured in standard medium supplemented with 1% pen (10×10^3 U/ml)-Strep (10 mg/ml) during 21 days to obtain fully differentiated cells. The cells were then washed with phosphate buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 1.14 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.2) and each treatment was applied for 24 h in culture medium containing 1% (v/v) FBS. In an additional experience, a selection of the treatments was applied on differentiated as well as proliferating cells; the latter consisting in Caco-2 cells treated 24 h after seeding.

2.3. Determination of cytotoxicity

Cytotoxicity of the treatments was evaluated by measuring the activity of lactate dehydrogenase (LDH) in the culture media, since this enzyme is released by damaged or necrotic cells (Cytotoxicity detection kit, Roche Diagnostics GmbH, Mannheim, DE). Results were expressed as a percentage of the positive control, consisting of cells exposed to 1% (v/v) Triton-X-100.

2.4. Determination of IL-6 and IL-8 secretion

The extracellular media were collected and centrifuged at 16×10^3 g for 10 min. IL-6 and IL-8 secretion was evaluated using a sandwich Elisa method (BD Biosciences Pharmingen, San Diego, CA) as in Van De Walle et al. (2008) and quantified in pg/ml using the standard provided with the kit, with sensitivity limits being of 2.2 and 0.8 pg/ml, respectively for the IL-6 and IL-8 assay. Results were subsequently expressed in relative terms to the negative control (untreated cells) to facilitate comparison between groups.

2.5. Determination of the arachidonic acid cascade activity through PGE-2 production

The activation of the arachidonic acid cascade was estimated through the production of PGE-2 after addition of arachidonic acid (Romier-Crouzet et al., 2009). Briefly, 24 h after treatment, cells were washed and incubated with 10 mM arachidonic acid in PBS during 10 min. PGE-2 secretion in extracellular medium was quantified in pg/ml using a Prostaglandin E_2 Enzyme Immunoassay Kit (Cayman Chemical Corporation, Inc. Ann Arbor, MI) in accordance with the manufacturer's protocol, with a LOD of 15 pg/ml. The amount of PGE-2 produced by untreated cells was subtracted from the other values to obtain the amount of PGE-2 produced by induction of the arachidonic acid cascade (further referred to as "induced PGE-2 production").

2.6. Determination of NO production

Nitric oxide, present in the culture medium as nitrite and nitrate, was assayed using the colorimetric Nitric Oxide Assay