

EFFECT OF ENTERIC FLORA ON INFLAMMATORY AND ANGIOGENIC MECHANISMS IN HUMAN INTESTINAL MICROVASCULAR ENDOTHELIAL CELLS

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1. ABSTRACT

The intestine is a highly vascularized organ, and the splanchnic microcirculation is now appreciated to play a key role in immune and inflammatory responses in the gut. Emerging evidence demonstrates that the enteric flora not only exerts an important effect on innate immunity and the mucosal immune system, but will also affect inflammatory and angiogenic mechanisms involving the gut microcirculation. In response to bacterial lipopolysaccharide, the intestinal microcirculation and its endothelial lining will undergo activation, which will contribute to cell adhesion molecule expression and the recruitment of leukocytes into the gut wall, an early and rate limiting step in the inflammatory process. This is balanced by the fact that human intestinal microvascular endothelial cells possess specific mechanisms of endotoxin tolerance, which will diminish inflammatory activation in response to repeated bacterial activation. Likewise, the process of angiogenesis, or new blood vessel growth is influenced by the presence of bacteria, which stimulate the release of angiogenic factors from innate immune mechanisms. Conversely, bacterial derived products of dietary carbohydrate fermentation, the short chain fatty acids, will decrease angiogenic mechanisms in human intestinal microvascular endothelial cells. In this review we summarize our present state of knowledge regarding the interplay between enteric flora and inflammatory and angiogenic activation of the intestinal microcirculation its microvascular endothelium.

2. INTRODUCTION

Recent investigation into the pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of chronic inflammatory bowel disease (IBD), has pointed to etiologic mechanisms involving the innate immune response to bacteria and their products. A convergence of data from genetic (1), environmental (2) and immunological studies (3) (4) suggests that chronic mucosal inflammation may result from a breakdown in mechanisms of endotoxin tolerance in the innate mucosal

immune system. Endothelial cells lining the microcirculation in the gut are now appreciated to play a central role in maintaining mucosal immune homeostasis and inflammation, and possess potent innate immune response mechanisms (5, 6). Following stimulation with bacterial lipopolysaccharide (LPS), microvascular endothelial cells will undergo activation, resulting in increased expression of cell adhesion molecules and chemokines, which will in turn mediate recruitment of circulating leukocytes into tissues and foci of inflammation. Because human gut microvascular endothelial cells exist in close proximity to vast numbers of enteric bacteria and their products, recent investigation has focused on defining specific mechanisms of "programmed hyporesponsiveness" or endotoxin tolerance which will prevent excessive, acute inflammatory activation and tissue damage. In addition, emerging data suggests that angiogenesis, or new blood vessel growth, an essential component of wound healing, is intimately linked to the presence of enteric bacteria and innate immune mechanisms (7). In this review, we discuss the specific innate immune mechanisms employed by human intestinal microvascular endothelial cells to react to enteric pathogens, as well as their ability to develop endotoxin tolerance in response to repeated LPS exposure. In addition, we also review the modulatory effect of bacterial derived fermentation products on gene expression and angiogenic mechanisms. Like epithelial cells which line the mucosal interface in the gastrointestinal tract, human gut specific microvascular endothelial cells also possess innate mechanisms to respond to the intestinal flora, as well as specific mechanisms to regulate excessive activation in response to these bacteria and their products.

3. HUMAN INTESTINAL MICROVASCULAR ENDOTHELIAL CELLS AND THE GUT MICROCIRCULATION

The human intestine is a highly vascularized organ, which will receive increased cardiac output in

response to physiologic need (8). The intestinal microvasculature is lined with a specialized single-cell layer of endothelium which regulates gut perfusion, playing a central role in essentially all of the physiologic functions of the intestine. In addition to the gut lumen, the intestinal microvasculature represents a second conduit which both enters and exits the intestine. The endothelial lining of the intestinal microcirculation plays a central role in the regulation of perfusion, which involves the local generation of nitric oxide (NO), and cyclo-oxygenase derived vasoactive prostaglandins (9).

In addition to its role in tissue perfusion, the microvascular endothelium plays a critical role in mucosal immune homeostasis. It is now clear that intestinal microvascular endothelial cells play a central role in trafficking of immune cells into the mucosal immune compartment. The identification of the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), has resulted in an improved understanding of the cellular and molecular mechanisms which underlie trafficking of immune cells into the gut. During normal physiology, immune cells expressing the alpha4 integrin will preferentially bind the MAdCAM-1 expressed on gut specific microvascular endothelial cells (10). During pathologic, chronic intestinal inflammation, MAdCAM-1 expression is markedly upregulated (11).

4. MICROVASCULAR ENDOTHELIAL CELLS AND THE INNATE IMMUNE SYSTEM

Among non-immune cell populations, microvascular endothelial cells play a dynamic and central role in the regulation of the mucosal immune response. Microvascular endothelial cells play a key regulatory role as "gate-keeper" of mucosal inflammation by regulating the recruitment and diapedesis of circulating immune cells into inflammatory sites. Recruitment of immune cells into the intestinal mucosa and submucosa are some of the most remarkable features of both acute and chronic mucosal inflammation. Our current understanding indicates that the process of leukocyte adhesion and emigration from the circulation involves multiple sequential steps of cell-cell adhesion and activation (12). The initial rolling interactions between leukocytes and endothelial cells prior to firm adhesion are mediated by the selectins, including E (endothelial)-selectin. This selectin-mediated tethering and rolling is a prerequisite step for the following integrin-mediated interactions, and is also considered essential for leukocyte activation by enhancing exposure to chemoattractants through prolonging contact with the vessel wall. Following rolling adhesion, firm adhesion and diapedesis appear to be mediated by the interaction between leukocyte integrins and the integrin-ligands, members of the immunoglobulin gene superfamily such as ICAM-1 expressed on endothelial cells. Using primary cultures of human intestinal microvascular endothelial cells, multiple research groups have defined that LPS stimulation will result in a rapid and significant increase in expression of the cell adhesion molecules E-selectin, ICAM-1, and VCAM-1 (13) (5) (14). In addition to these cell adhesion molecules, intestinal microvascular endothelial cells produce a number of chemokines (e.g. IL-

8, MCP-1) or acute phase response cytokines (e.g. IL-6), contributing to aggregation and activation of inflammatory cells. Again, stimulation of HIMEC with LPS will result in marked increases in expression of these molecules (14). Investigation of HIMEC activation has demonstrated that the response to LPS activation is similar to that induced by the pro-inflammatory cytokines TNF-alpha or interleukin 1beta, which are both associated with severe destructive inflammation in IBD. Because HIMEC will typically exist in close proximity to large quantities of bacteria, the propensity for these cells to undergo chronic and auto-destructive inflammatory activation suggested that specific mechanisms to limit endotoxin activation would be required in this cell population to maintain homeostasis.

During health, enteric bacteria will not induce a destructive inflammatory response from the mucosal immune system, but instead will exist in a state of "physiologic inflammation" within the gut mucosa (15). When the gut barrier function is disrupted as a result of enteric pathogenic infection or potentially as a result of a dys-regulated immune response towards luminal bacteria, further inflammatory destructive effects may result. It is therefore highly likely that the microcirculation in the intestine may come into immediate proximity with enteric organisms. In this scenario, activation of microvascular endothelial cells by LPS will rapidly amplify and upregulate inflammation, resulting in the increase of the recruitment and activation of immune cells. Those immune cells further produce pro-inflammatory immune-modulators (i.e. TNF-alpha, IL-1beta, etc.), which will in turn further activate the endothelium. Thus, the gut microvascular endothelium and mucosal immune cells will comprise a positive feedback system to amplify and exacerbate inflammation, which can be both physiologic and pathologic.

Work using purified primary cultures of HIMEC has defined many of the activation mechanisms in these cells. HIMEC will undergo the most potent inflammatory activation in response to inflammatory and immunomodulatory cytokines (TNFalpha, IL-1beta, TGFbeta, etc.), bacterial products (endotoxin; LPS). Likewise, the limitation of inflammatory activation in HIMEC is influenced by molecules which are increased during the inflammatory process in the gut, specifically nitric oxide (NO) (5, 16, 17). The generation of iNOS derived NO following inflammatory activation in response to pro-inflammatory cytokines or LPS, is the most potent downregulatory mechanism that we have identified in the acute activation of HIMEC (17). Thus, human gut microvascular endothelial function appears to be finely regulated by the effect of inflammatory mediators, which will mediate the upregulation of the inflammatory response as well as its subsequent programmed downregulation through NO dependent mechanisms.

5. MECHANISMS OF ENDOTOXIN TOLERANCE IN HIMEC

Among the activators of endothelial cells described above, some bacterial products cause significant

pro-inflammatory activation in endothelial cells. The innate immune response to LPS is a critical mechanism for host-defense, which will induce cell adhesion molecule expression, NO production, chemokine/cytokine production and increased leukocyte binding. However, sustained inflammatory activation in response to LPS may also lead to damaging effects, and is believed to play a major etiopathogenic role in endotoxic shock, multi-system organ failure and inflammatory bowel disease (18, 19). Recent studies in our laboratory suggest that intestinal endothelial cells possess specific mechanisms to decrease sustained inflammatory responses toward chronic endotoxin exposure. Using primary cultures of HIMEC, repeated LPS exposure demonstrated a down-regulation of inflammatory activation, characterized by impaired IL-6 and E-selectin expression, diminished superoxide production, impaired MAPKs activation and decreased leukocyte binding (6, 20, 21). The prototypical LPS receptor Toll-like receptor 4 (TLR4) is abundantly expressed in HIMEC, but did not undergo modulation in response to LPS exposure, which did not contribute to the phenomenon of endotoxin tolerance. HIMEC failed to express TLR2, and were dependent on the presence of serum derived CD14 to undergo activation in responses to LPS. In summary, endothelial cells demonstrate "endotoxin tolerance", the LPS-induced transient impaired inflammatory response to subsequent LPS challenge which has been described most extensively in monocytes and macrophages (22).

A major mechanism which downregulates the inflammatory activation of HIMEC involves the generation of NO via the high output enzymatic pathway. Following activation in response to LPS, TNF-alpha or IL-1beta alone or in combination, HIMEC will increase NO production, which corresponds with transcription of the NOS2 (iNOS) isoform. This increased endothelial production of NO may function as an endogenous antioxidant, which rapidly reacts with intracellular oxyradical species which are rapidly generated following cytokine and LPS induced activation in HIMEC. Increased endothelial generation of superoxide anion measured in HIMEC with hydroethidine intravital staining, is linked to inflammatory activation, and the increased generation of NO is believed to quench this oxyradical. In our experiments investigating endotoxin tolerance in HIMEC, we found that repeated rounds of LPS activation resulted in a progressive decrease in superoxide anion. What correlated with this decrease in superoxide generation was a progressive increase in antioxidant defense, specifically the enzyme Manganese superoxide dismutase.

6. SPECIFIC ENTERIC PATHOGENS AND HIMEC

The enteric microcirculation is heavily damaged by entero-hemorrhagic infections, which include shigella, salmonella, enterotoxigenic *E.coli*. Mechanistically, the toxins produced by these organisms will target and interact with specific cells in the gastrointestinal mucosa. Shiga toxin is the prototypical enterotoxin and is produced by a subgroup of enteropathogenic *Escherichia coli*, referred as Shiga-toxin producing *Escherichia coli* (STEC). STEC such as *E. coli* O157:H7 are an important cause of haemorrhagic colitis and the diarrhea-associated form of the haemolytic uraemic syndrome (23). Human intestinal

microvascular endothelial cells constitutively express Shiga toxin receptors at high levels for both Shiga toxin 1 and 2, and are sensitive to these toxins (24). Recent report showed that Shiga toxin 1 and/or 2 induce cell adhesion molecules (ICAM-1), chemokines (GRO, IL-8) or cytokine (IL-6, MCP-1) expression in endothelial cells (25), indicating that Shiga-toxin elicits pro-inflammatory response of this cell population.

Additional innate immune response mechanisms which are specific to enteric pathogens, include TLR5, the flagellin receptor. Salmonella enteritis is associated with epithelial disruption which leads to bacterial translocation into the submucosa. Expression of the TLR5 receptor by HIMEC represents an innate immune defense mechanism in response to this pathogen (26).

7. PRODUCTS OF BACTERIAL FERMENTATION AND ACTIVATION OF HIMEC

In addition to direct effects of bacterial components on endothelial innate immune mechanisms, bacterial derived products from the luminal gastrointestinal tract will also exert potent effects on mucosal cell populations. Although the majority of bacteria/bacterial derived products may be regarded as pro-inflammatory factors in mucosal immune homeostasis, there are additional factors derived from the enteric flora which play a key role in maintaining mucosal homeostasis. One of major groups of bacterial derived homeostatic factors are the short chain fatty acids (SCFAs) derived from enteric bacterial fermentation of non-digested dietary carbohydrates. Sodium butyrate is the major SCFA derived from bacterial fermentation, and this compound provides not only an essential energy substrate for the colonic epithelium and also regulates epithelial immune functions, which include the induction of differentiation, inhibition of cell proliferation, induction of apoptosis (27-30), suppression of IL-8 secretion (31), and inhibition of NFkappaB activation by TNFalpha or PMA (32). In addition to their effect on epithelial cells, sodium butyrate also has the capacity to influence the homeostatic and immune functions of human intestinal endothelial cells. Recent investigation has demonstrated that sodium butyrate has the capacity to differentially modulate gene expression in HIMEC. Sodium butyrate will diminish the proinflammatory effect of LPS, by decreasing IL-6 and cyclooxygenase-2 (COX-2) expression, while it will paradoxically increase the expression of ICAM-1 following endotoxin activation (14). Physiologically, sodium butyrate exerted a potent effect inhibiting VEGF induced angiogenesis in HIMEC. The mechanism of sodium butyrate's ability to modulate gene expression appeared to function through the inhibition of histone deacetylase. Thus, in addition to endogenous regulators such as cytokines, innate immune response of endothelial cells toward luminal bacteria is also regulated by bacterial products in a complex manner.

8. SUMMARY

In summary, the human gut specific microvascular endothelium exists in a balance with enteric bacteria and their products. HIMEC possess specific

Table 1. The effects of repeated LPS stimulation and sodium butyrate on LPS- induced gene and/or protein expression (6, 14).

Gene and/or protein expression	Single stimulation LPS	Effect of repeated LPS stimulation	Effect of butyrate on LPS-induced expression
ICAM-1	↑↑	→	↑
E-selectin	↑↑	↓	→
VCAM-1	↑↑	↓	N.A
IL-6	↑↑	↓	↓
IL-8	↑↑	→	→
HLA-DR	↑↑	→	N.A
CD86	↑↑	↓	N.A
Mn-SOD	↑↑	↑	→
COX-2	↑↑	N.A	↓
Leukocyte binding	↑↑	↓	N.A

(N.A = no data available)

Table 2. The effects of repeated LPS stimulation and sodium butyrate on LPS- induced activation of NFkappaB and intracellular signaling mechanisms in HIMEC (6, 14).

Signaling mechanisms	Single stimulation LPS	Effect of repeated LPS stimulation, early, late	Effect of butyrate on LPS-induced activation
NFkappaB	↑↑	↓ →	→
p38 MAPK, p44/42MAPK, JNK	↑↑	↓ ↓↓	N.A
superoxide anion	↑↑	↓ ↓	N.A

mechanisms to react rapidly to LPS induced stimulation, but will rapidly adapt to repeated LPS challenge by downregulating intracellular mechanisms of activation involving MAPK activation and superoxide generation. Likewise, bacterial derived fermentation products from non-digestible dietary carbohydrate, including sodium butyrate, exert potent effects on HIMEC gene expression. As summarized in Tables 1 and 2, the patterns of activation and gene expression seen in HIMEC exposed to both chronic LPS activation as well as bacterially derived luminal products are markedly different from the patterns of acute activation. These findings suggest that further investigation needs to consider the complex interplay between the innate immune mechanisms in various mucosal cellular constituents, as well as the prolonged exposure of bacteria and their products when modeling mucosal immune function. Further work into the microvascular biology of HIMEC will help in defining the mechanisms underlying dysregulated chronic inflammation in IBD, as well as the impaired wound healing which characterizes these disorders.

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