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Bacterial DNA Induces Pulmonary Damage via TLR9 through Cross-Talk with Neutrophils

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Abstract

Bacterial DNA (bDNA) contains hypo-methylated “CpG” repeats that can be recognized by toll-like receptor (TLR)-9 as a pathogen-associated molecular pattern (PAMP). The ability of bDNA to initiate lung injury via TLR-9 has been inferred on the basis of studies using artificial CpG DNA. But the role of authentic bDNA in lung injury is still unknown. Moreover, the mechanisms by which CpG DNA species can lead to pulmonary injury are unknown, although neutrophils (PMN) are thought to play a key role in the genesis of septic acute lung injury (ALI). We evaluated the effects of bDNA on PMN-endothelial cell (EC) interactions thought critical for initiation of ALI. Using a bio-capacitance system to monitor real-time changes in endothelial permeability, we demonstrate here that bDNA causes EC permeability in a dose-dependent manner uniquely in the presence of PMN. These permeability changes are inhibited by chloroquine, suggesting TLR9-dependency. When PMN were pre-incubated with bDNA and applied to EC or when bDNA was applied to EC without PMN, no permeability changes were detected. To study the underlying mechanisms we evaluated the effects of bDNA on PMN-EC adherence. bDNA significantly increased PMN adherence to EC in association with up-regulated adhesion molecules in both cell types. Taken together, our results strongly support the conclusion that bDNA can initiate lung injury by stimulating PMN-EC adhesive interactions predisposing to endothelial permeability. bDNA stimulation of TLR9 appears to promote enhanced gene expression of adhesion molecules in both cell types. This leads to PMN-EC cross-talk which is required for injury to occur.

Keywords

Pathogen Associated Molecular Patterns (PAMPs); CpG DNA; bacterial DNA; lung injury; vascular permeability; ECIS; Adherence; qPCR

INTRODUCTION

Neutrophils (PMN) play an essential role in infection and innate immunity, both providing early defense against invading microorganisms or initiating deleterious SIRS responses (1,

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2). PMN comprise approximately two-thirds of peripheral blood leukocytes and transit rapidly to sites of infection. There, they migrate down chemotactic gradients to limit infection and allow recruitment and activation of other immune cells through the release of inflammatory mediators and antimicrobial products. This results in pathogen clearance and ultimately, in the initiation of an adaptive immune response (3). Conversely, excessive or inappropriate PMN activation result in inflammation that causes tissue injury and contribute to the initiation of a variety of noninfectious illnesses including rheumatoid arthritis, inflammatory bowel disease, asthma or chronic obstructive pulmonary disease. In the setting of acute sepsis however, the most feared complication of PMN activation is Acute Lung Injury (ALI), which can lead to Acute Respiratory Distress Syndrome (ARDS) (4, 5).

The toll-like receptors (TLRs) are a family of innate immune receptors that can recognize conserved pathogen associated molecular patterns (6). Recognition of such 'Danger' signals (7) by the TLRs leads to activation of a group of overlapping signaling systems that utilize the adaptor molecule MyD88. These systems appear to play a central role in the inflammatory responses to sepsis. The prototype for TLR activation in sepsis is immune cell stimulation by endotoxin via TLR4 (8). Less is known about how other bacterial PAMPs activate immune cells although bDNA is thought to activate innate immunity by binding to the endocytic receptor TLR-9 (9).

To date, studies (10-12) evaluating the role of TLR9 in sepsis and lung injury have focused on the interactions of TLR9 with artificial CpG DNAs. These are synthesized to have hypomethylated areas that bind TLR9. No studies have assessed the effects of authentic bacterial DNA (bDNA) on human immune cells and moreover, no studies have examined how bDNA-induced events might cause interactions between immunocytes and the lung that could contribute to the pathogenesis of ALI/ARDS.

We hypothesized that if bDNA contributed to lung injury, it was likely to activate PMN and / or pulmonary endothelial cells (EC), promoting PMN-EC adherence and interactions that led to increased endothelial permeability. We therefore investigated whether and how bDNA acted on PMN and EC in co-culture and affected the permeability of EC-monolayers.

MATERIALS AND METHODS

Compliance

All studies performed were approved by the IRB of Beth Israel Deaconess Medical Center. Human blood specimens were obtained from volunteer donors by venipuncture after informed consent.

Endothelial Cell cultures and chemicals

EA.hy926 cells, derived by the fusion of HUVECs with the continuous human lung carcinoma cell line A549, were obtained from Dr. Cora-Jean C. Edgell (University of North Carolina at Chapel Hill, NC) and maintained in DMEM medium with 10% FBS and penicillin and streptomycin in a CO₂ (5%) incubator at 37°C (13). Bacterial DNAs (single or double stranded) were purchased from Sigma (St. Louis, MO) or Invivogen (San Diego, CA). Bacterial DNAs were endotoxin-free. Human TLR9 specific inhibitor, ODN TTAGGG, and an inhibitor for endosomal acidification, chloroquine, were purchased from Invivogen. Other chemicals were purchase from Sigma.

Expression of ICAM-1, E-selectin, and TLR9 in EA.hy926 cells

EA cells grown in 6 well plates were treated with bacterial DNA (10 or 20 µg/mL), LPS (100 ng/mL), or medium for 6 hrs at 37°C in 5% CO₂. RNA and cDNA were prepared from

the cells using RNeasy (Qiagen, Valencia, CA) and VILO (Invitrogen, Carlsbad, CA), kits respectively, following the manufacturers' protocols. RNA and cDNA concentrations were determined by the Nanodrop (Thermo Fisher Scientific, Hudson, NH). Gene expression levels of ICAM-1, E-selectin, and TLR9 were assayed by qPCR (Realplex, Eppendorf, Hauppauge, NY) using pre-validated specific primers from OriGene (Rockville, MD). Data were normalized using GAPDH as the housekeeping gene and gene expression by medium-treated samples was used as 100%.

Gene expression of CD11b, CD18, and TLR9 in human PMN

Freshly isolated PMN were seeded on 6 well-plate and were treated with bacterial DNA (10 or 20 $\mu\text{g/mL}$), LPS (100 ng/mL), or medium for 90 min at 37°C, 5% CO_2 incubator. As shown above, qPCR for CD11b, CD18, and TLR9 was performed. Data were normalized as described above. In addition, CD11b expression was evaluated using FACS (FACSCalibur, BD Bioscience, Chicago, IL) with specific CD11b antibody by following manufacturer's protocol.

Human PMN preparations

PMN were isolated fresh from healthy volunteer donor blood. Detailed methods for PMN preparation can be found elsewhere (14). Briefly, PMN were isolated from minimally heparinized whole blood using a one-step centrifugation procedure on PMN Isolation Medium (Thermo Fisher Scientific, Hudson, NH). The neutrophil layer was collected and osmolality was restored. Cells were then washed and suspended in HEPES buffer. Residual red blood cells were lysed briefly to increase PMN purity.

Human PMN-Endothelial Cell adhesion assay

PMN were incubated for 30 minutes at 37°C in the dark in HEPES buffer containing 1mM Ca^{2+} and 3 μM Calcein-AM. Calcein-labeled PMN were set aside for use as a standard curve and PMN-endothelial cell adhesion assays were performed as previously described (15, 16). Near confluent EC monolayers were incubated with E.coli DNA (10-20 $\mu\text{g/mL}$), LPS (100 ng/mL) or medium for 6 hours with 2.25×10^5 PMN, after which the plates were inverted and centrifuged at 200g for 5 min at room temperature to remove non-adherent PMN. Calcein fluorescence of neutrophils was measured using a fluorescent plate reader (SpectaMax: Molecular Devices, Sunnyvale, CA) set at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

EC permeability measurements

Transendothelial Electrical Resistance (TER) of EC monolayers was measured by seeding $2-3 \times 10^5$ EA hy.926 cells onto cysteine and fibronectin-pretreated 8W10E+ cultureware (40 electrodes per well) and incubated (37°C, 5% CO_2) in an ECIS system (Applied BioPhysics, Troy, NY) (17, 18). This system measures changes in impedance of the monolayer to AC current flow. The micro-currents used have no detectable effect on the cells (17). Confluence was determined by capacitance at 64 kHz coming to a plateau ($\sim 10\text{nF}$) after several hours of incubation (17). In this system impedance decreases and capacitance increases as permeability increases (17, 19). EC permeability change was assessed as capacitance change at 64 KHz (19) after addition of 1) media, 2) human PMN (2×10^5) freshly isolated from healthy volunteers, 3) bacterial DNA or 4) bacterial DNA and PMN. Capacitance values in each well were normalized to capacitance at the start of treatments. Finally, values from each microelectrode were pooled at discrete time points and plotted versus time as the mean $\pm$ SE.

Results

Bacterial DNA increases endothelial permeability

Increased endothelial permeability links immunologic ALI to the altered lung mechanics and impaired gas exchange seen in ARDS. We hypothesized that authentic E.coli DNA (bDNA); acting either alone or through the agency of activated PMN, might act on EC monolayers to increase their permeability. As shown in Figure 1A, untreated EA cells or cells having only a medium change at T=0 show no permeability changes over the time course studied. When the EA cells without PMN were treated with CpG DNA (1, 10 or 20 $\mu\text{g/mL}$) there was no significant change in permeability seen. Similarly, PMN exposed to bDNA and then applied to the EC failed to induce a permeability change (data not shown). But when PMN were applied to confluent EC and bDNA was added to the intact system we saw brisk, dose-dependent increases in permeability (Figure 1A). Similar to CpG, when E.coli DNA (10 or 20 $\mu\text{g/mL}$) was applied to confluent EA cells, permeability was increased only in the presence of PMN (Figure 1B). However, E. coli DNA seems to be effective at the lower concentrations (10 $\mu\text{g/mL}$) than the higher concentrations (20 $\mu\text{g/mL}$).

Bacterial DNA increases PMN-EA adherence

PMN adhesion to endothelial cells is an important first step on the recruitment of PMN to areas of active infection. Adherence to activated EC is a pre-requisite for PMN transmigration into the alveoli and PMN-EC interactions during adherence and transmigration can lead directly to increased vascular permeability (19). PMN were applied to endothelial monolayers in the presence of E. coli DNA or medium and assayed for adherence. LPS (100 ng/mL) served as a positive control. As shown in Figure 2, bDNA (10-20 $\mu\text{g/mL}$) significantly increased the adherence of calcein-loaded PMN to EC as compared to medium treated controls (One Way ANOVA, $p < 0.001$).

Bacterial DNA increases adherence molecule gene expression in both EC and PMN

Since bDNA increased adherence of PMN to EA monolayers we evaluated whether it affected expression of the molecules that cause those cells to adhere. Thus we incubated EA cells (6h) or human PMN (90 min) with bDNA at various concentrations and performed qPCR to determine gene expression levels of endothelial ICAM-1 and E-selectin. TLR9 expression was also assessed using specific primers. Data were normalized to GAPDH levels with expression after medium treatment used as a negative baseline control. Response to LPS was used as a positive control. As shown in Figure 3, treatment with bDNA increased EC expression of ICAM-1 and E-selectin in a dose-dependent fashion. No change was detected in the expression of TLR9. We then evaluated PMN CD11b and CD18 expression after bDNA treatment. In Figure 4A we see that bDNA **markedly** increases PMN expression of CD11b and CD18 whereas TLR9 expression is unchanged. Similarly, CD11b increase in PMN by bDNA treatment was detected by FACS using human CD11b specific antibody (Figure 4B).

Endothelial permeability increase by bacterial DNA is TLR9-dependent

Prior studies have shown that CpG DNA can act on immunocytes via TLR9. Chloroquine (CQ) is an inhibitor of TLR interactions with CpG DNAs and also of endosomal acidification. To provide proof-of-concept for in vivo therapeutic studies, we studied whether CQ inhibition could inhibit bDNA-induced increases in permeability in our PMN-EC co-culture systems. As clearly shown in Figure 5A, the increased in endothelial permeability that was caused by bDNA in the presence of PMN was markedly attenuated by CQ. CQ had no direct effect on EC permeability.

Furthermore, the permeability increase caused by single stranded E.coli DNA (ssE.coli DNA, 10 $\mu\text{g/mL}$) was inhibited in the presence of human TLR9 specific inhibitor, ODN TTAGGG (Figure 5B). This inhibitor may slightly increase barrier integrity although it may not be significant.

DISCUSSION

Endothelial cell (EC) permeability is often pathologically increased in sepsis and related inflammatory conditions. This process plays a central role in initiating septic acute lung injury (ALI) and its progression to acute respiratory distress syndrome (ARDS). EC can be activated either through the actions of plasma-borne mediators of inflammation and coagulation, or through cell-cell interactions with immunocytes like PMN. It is well known that release of bacterial PAMPs in sepsis can activate EC through ligation of TLR2 or 4 by LPS (20). A similar role has more recently been postulated for bacterial DNA since it can activate immune cells via TLR9. No direct studies exist however, assessing whether bDNA does in fact, activate interactions of WBC and EC that lead to increased capillary permeability, and if so, what mechanisms are involved.

We studied the contribution of bDNA to lung injury in sepsis by assaying its effects on EC monolayer permeability. The ECIS system allows real-time study of changes in monolayer impedance and capacitance that reflect resistance and permeability respectively (17, 19, 21). Interestingly, we found that applying bDNA directly either to the endothelial monolayers or to PMN prior to co-culture with the EC failed to result in any changes in permeability. In distinction, exposure to G-protein ligands like thrombin or histamine cause immediate increases in EC permeability (19). But when PMN and EC were stimulated in co-culture, the presence of bDNA resulted in dose-dependent increases in EC monolayer permeability (Figure 1A, B). Thus bacterial DNA does not increase EC permeability by isolated effects either on PMN or EC. Rather, direct physical interaction between bDNA-activated PMN and bDNA-activated EC is an absolute requirement for bDNA to mediate EC permeability.

EC-WBC interactions that lead to permeability changes typically begin with adherence of the WBCs to the endothelium (22). So next we examined whether increased permeability was associated with increased PMN adherence to the EC monolayer and we found that bDNA did enhance PMN adherence to EA cell monolayers (Figure 2). Finding that to be the case, we next used qPCR to examine whether the enhanced adherence of PMN to EC after bDNA exposure reflected up-regulation of adhesion molecule gene expression on the EC and/or PMN. As shown in Figure 3, EC gene expression of selectins was strongly increased by incubation with bDNA. Selectins however, only initiate neutrophil 'rolling'. Strong adhesion and transmigration are initiated by interactions of the PMN integrin CD11b/CD18 with ICAM-1 on the endothelial surface. Gene expression of each of these molecules was enhanced by exposure to bacterial DNA. In contrast, TLR 9 expression level was unchanged by exposure to bDNA (Figure 3, 4A, 4B).

The results above all suggest that bacterial DNA increases PMN-endothelial cell adherence by increasing expression of adherence molecules on endothelial cells and PMN. Bacterial DNA is thought to activate inflammation through ligation of the endosomal receptor TLR9. Indeed, lung injury caused by artificial CpG DNA depends upon the presence of TLR9 receptors (10).

We sought to study the mechanisms underlying the regulation of adherence molecules and also to begin evaluation of potential therapeutic means of preventing bDNA mediated increases in EC permeability. We therefore applied the TLR9 inhibitor chloroquine (5 μM) to ECs during ECIS experiments. As before, when EC and PMN were stimulated together

by bDNA endothelial permeability increased. As seen in Figure 5 however, this increase was inhibited by the presence of chloroquine where chloroquine itself had no direct effect on permeability. Furthermore, human TLR9 specific inhibitor, ODN TTAGGG, inhibited permeability increase caused by ssE.coli DNA (Figure 5B). These results suggest that the increased EC permeability elicited by bDNA in the presence of PMN was TLR9 dependent and that chloroquine and TLR9 specific inhibitors might limit pathologic expression of adherence molecules on EC and PMN in sepsis.

It was especially interesting that neither medium pre-conditioned by PMN treated with bacterial DNA, nor co-culture with freshly isolated PMNs treated with bDNA increased EC permeability when exposed directly to them. The only time that EC permeability increased was when EC and PMN were exposed to bDNA together. This finding demonstrates cross-talk between EC and PMN wherein bDNA leads indirectly to cell activation through paracrine cellular interactions. Identification of the mechanisms involved in these direct interactions will be the subject of future research.

In any case, the events leading to permeability were at least partially inhibited by the presence of chloroquine. This suggests that the process is TLR9-dependent. El Kebir et al. recently showed that bacterial DNA activates HUVEC directly, increasing the expression of adhesion molecules and causing PMN adherence (23). Our results using EA hy.926 cells are similar but in addition we were able to show that bDNA could increase endothelial permeability only when EC and PMN were stimulated together. *In vivo* studies by Yamada et al have also suggested the importance of this pathway by showing CpG-induced lung inflammation in the mouse was inhibited by oligodeoxy-nucleotides (ODNs) that prevent CpG binding to TLR9 (12). Taken together, these findings suggest bDNA is a critical mediator of PMN-mediated septic lung injury and support the hypothesis that the use of chloroquine/TLR9 specific inhibitors could lead to a unique therapy for sepsis.

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Figure 1. Bacterial DNA induces EA permeability changes in the presence of PMN
 EA cells pre-seeded onto ECIS cultureware (8W10E+) were stimulated at $t=0$ (treatments).
A. CpG (1, 10, 20 $\mu\text{g/mL}$) or **B.** E.coli DNA (10, 20 $\mu\text{g/mL}$) were applied in the presence or the absence of PMN ($2 \times 10^5/\text{well}$). Data were normalized by the capacitance values (64,000Hz) at $t=0$. Data show mean and SD values from at least two wells (80 electrodes).

Figure 2. E.coli DNA increases EA-PMN adherence

EA cells pre-seeded on 96 well-plate were treated with E.coli DNA (10 or 20 $\mu\text{g/mL}$) or LPS (100 ng/mL) for 6 hrs. The freshly isolated human PMN (2×10^5) loaded with calcein were applied to EA cells for 60 min and then the plates were inverted and centrifuged at 200 g for 5 min. Adherent PMN were counted based on calcein fluorescence. Data were analysed as PMN adherence by %-medium-treated control. Mean and SE values from $n=8$ are shown.

Figure 3. Bacterial DNA increased adherence molecule gene expression in EA cells

EA cells were treated with E.coli DNA (10, 20 $\mu\text{g/mL}$) for 6 hrs and then RNA/cDNA were prepared using RNeasy and VILO cDNA synthesis kit from Qiagen and Invitrogen, respectively. ICAM-1, E-selectin, and TLR9 gene expression levels were evaluated using GAPDH as a house keeping gene. Levels of gene expression were shown as %-medium control. Mean and SE values are shown (n=3).

Figure 4. Bacterial DNA increased integrin molecules in PMN

A. PMN were treated with 1. medium (control), 2. CpG-10 (10 $\mu\text{g/mL}$), or 3. CpG-20 (20 $\mu\text{g/mL}$) for 90 min in DMEM. Then RNA/cDNA were as described in Figure 3. CD11b, CD18 and TLR9 gene expression levels were evaluated using GAPDH as a house keeping gene. Levels of gene expression were shown as %-medium control. Mean and SE values are shown (n=3). * denotes statistical significance. **B.** PMN were stimulated with medium (control) or E.coli DNA (10 $\mu\text{g/mL}$) for 1 hr. Then PMN were incubated with CD11b-FITC antibody followed by FACS analysis following the manufacturer's protocol. CD11b expression was shown by %-control.

Figure 5. Chloroquine and TLR9 specific inhibitor inhibit bacterial DNA-induced permeability increase

A. CpG (20 $\mu\text{g/mL}$) was applied to EA cells seeded on ECIS cultureware (8W10E+) with PMN (2×10^5). The presence of chloroquine (5 μM) inhibited CpG-induced permeability increase by 50%, however chloroquine itself did not have any effects on endothelial permeability. Mean and SD values for at least two wells with 80 electrodes are shown except medium treatment ($n=1$, 40 electrodes). **B.** Similar to **5A**, ssE.coli DNA (10 $\mu\text{g/mL}$) was applied to EA cells. The presence of human TLR9 specific inhibitor, 5 μM ODN TTAGGG, completely inhibited permeability increase caused by E.coli DNA. Mean and SD values for two wells with 80 electrodes are shown. ODN may slightly increase barrier integrity although it may not be significant (data not shown).