Activated macrophages inhibit enterocyte gap junctions via the release of nitric oxide

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Am J Physiol Gastrointest Liver Physiol 294: G109–G119, 2008. First published November 1, 2007; doi:10.1152/ajpgi.00331.2007.—Enterocytes exist in close association with tissue macrophages, whose activation during inflammatory processes leads to the release of nitric oxide (NO). Repair from mucosal injury requires the migration of enterocytes into the mucosal defect, a process that requires connexin43 (Cx43)-mediated gap junction communication between adjacent enterocytes. Enterocyte migration is inhibited during inflammatory conditions including necrotizing enterocolitis, in part, through impaired gap junction communication. We now hypothesize that activated macrophages inhibit gap junctions of adjacent enterocytes and seek to determine whether NO release from macrophages was involved. Using a coculture system of enterocytes and macrophages, we now demonstrate that “activation” of macrophages with lipopolysaccharide and interferon reduces the phosphorylation of Cx43 in adjacent enterocytes, an event known to inhibit gap junction communication. The effects of macrophages on enterocyte gap junctions could be reversed by treatment of macrophages with the inducible nitric oxide synthase (iNOS) inhibitor L-Lysine or acetamidino hydrochloride (t-NIL) and by incubation with macrophages from iNOS−/− mice, implicating NO in the process. Activated macrophages also caused a NO-dependent redistribution of connexin43 in adjacent enterocytes from the cell surface to an intracellular location, further suggesting NO release may inhibit gap junction function. Treatment of enterocytes with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) markedly inhibited gap junction communication as determined using single cell microinjection of the gap junction tracer Lucifer yellow. Strikingly, activated macrophages inhibited enterocyte migration into a scraped wound, which was reversed by t-NIL pretreatment. These results implicate enterocyte gap junctions as a target of the NO-mediated effects of macrophages during intestinal inflammation, particularly where enterocyte migration is impaired.

In response to epithelial mucosal injury, the process of healing is initiated by the migration of enterocytes from healthy areas to sites of mucosal disruption. This phenomena, termed “intestinal restitution,” leads to the rapid restoration of mucosal continuity (1, 42). Studies from our laboratory have demonstrated that intestinal restitution is significantly impaired during conditions of acute and chronic intestinal inflammation, such as neonatal necrotizing enterocolitis (8, 43), and during conditions of elevated NO release (9). To understand the mechanisms that govern intestinal restitution, both under basal conditions and after stimulation with cytokines such as NO, we have focused on the molecular interactions that exist between adjacent enterocytes within the mucosal lining. Specifically, adjacent enterocytes are interconnected by both tight junctions and gap junctions (2, 12). Tight junctions are also connected via gap junctions, intercellular channels that are composed of hexameric arrays of individual proteins zona occludens, claudin, and others (11, 24), which may oxidize sulfhydryl and thiol moieties and trigger cytotoxic processes including lipid peroxidation and DNA damage (24, 32). Although it is clear that macrophage-enterocyte interactions play an important role during intestinal inflammation, the precise molecular target within the enterocyte during the setting of mucosal injury and the involvement of NO release, if any, in this process remain incompletely understood.

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connexin molecules that facilitate the passage of ions and solutes between adjacent cells (2, 10, 19). Studies from our laboratory have also shown that gap junction communication plays a pivotal role in the regulation of enterocyte migration and that inhibition of the phosphorylation of connexin43 (Cx43) leads to a loss of gap junction communication and the cessation of mucosal healing (37). We now seek to expand upon these recent observations to determine whether activated macrophages may alter the ability of adjacent enterocytes to undergo migration, and if so, whether the release of NO from such activated macrophages may inhibit enterocyte gap junctions in the process.

We now demonstrate that macrophages exert a striking effect on the gap junction properties of adjacent enterocytes. Specifically, we show that activated macrophages cause a striking reduction in the extent of phosphorylation and surface localization of the gap junction protein Cx43 in adjacent enterocytes and a reversible inhibition of enterocyte migration. Moreover, we now demonstrate that the inhibitory effects of macrophages on enterocyte gap junctions and the ability of enterocytes to migrate occur through the release of NO. These findings suggest a mechanism by which macrophage-enterocyte interactions participate in the pathogenesis of barrier dysfunction during intestinal inflammation.

MATERIALS AND METHODS

Cell culture and reagents. J774 macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum, 1% glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO₂ atmosphere as described (22). Intestinal epithelial cell (IEC)-6 cells were also obtained from the ATCC and maintained in DMEM supplemented with 5% fetal bovine serum, 1% glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1% insulin at 37°C in a 5% CO₂ atmosphere as described (7). Antibodies against phosphorylated Cx43 were from Chemicon (Temecula, CA); CD45 and phospho-CD45 antibodies were from Proteintech (Chicago, IL); and other antibodies were from Abcam (Cambridge, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless specified.

For the assessment of enterocyte-macrophage interactions, a coculture technique was adopted, as is depicted in Fig. 1. IEC-6 enterocytes were plated on the bottom well of six-well plates at 70–90% confluence, whereas J774 macrophages were plated onto a Transwell insert (0.4-μm pore size; Corning, Corning, NY). Cells were plated separately overnight, and macrophages were induced to become “activated” over 12 h by using a combination of LPS (10 ng/ml, Sigma-Aldrich) and IFN (100 U/ml, Sigma-Aldrich) in the presence or absence of the inducible nitric oxide synthase (iNOS) inhibitor l-lysine o-acetamidine hydrochloride (l-NIL, Sigma-Aldrich, 10 μM for 12 h). Cells were then washed to remove the LPS and IFN and combined in coculture for an additional 12 h, after which time cells were harvested and analyzed by immunofluorescence microscopy or SDS-PAGE.

For measurement of supernatant nitrite concentration, a Griess reaction was performed as described (20) on supernatant that had been aspirated from the top well of the Transwell filter after 12 h by using 1% sulfanilamide and 0.1% naphthylethylenediamine. Colorimetric analysis was performed at 550 nm.

For measurement of enterocyte proliferation, IEC-6 cells were plated to 60% confluence in the presence of media alone, unstimulated macrophages, and “activated” macrophages and then assessed using a colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (XTT) proliferation assay (Sigma-Aldrich) with 5 mg/ml XTT and absorbance measured at 450 nm according to the manufacturer’s instructions.

SDS-PAGE and immunofluorescence. SDS-PAGE was performed as described (8). Briefly, samples were solubilized in Laemmli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis using the Protean II minigel system (Bio-Rad), and transferred onto nitrocellulose membranes. Membranes were then immersed in blocking buffer (2% fish gelatin, 0.1% Tween) for 1 h. Blots were incubated with primary antibody solution overnight at 4°C at a 1:1,000 titer. Secondary antibodies were used at a concentration of 1:5,000. Bands were detected with enhanced chemiluminescence reagent (ECL-Super Signal; Pierce, Rockford, IL), and images were developed on radiographic film and quantified using a GS700 Bio-Rad densitometer and QuantityOne analysis software (Hercules, CA).

For immunofluorescence microscopy, IEC-6 cells were plated on top of 18-mm coverslips (Thermo Fisher Scientific, Waltham, MA) that were placed into the bottom of the six-well plates. All experiments were performed at 37°C in a humidified incubator with 5% CO₂ and 95% oxygen. Where indicated, IEC-6 cells were pretreated with nitric oxide donors S-nitroso-N-acetylpenicillamine (SNAP) or diethylaminomintamine-NONOate (detaNONOate) (Cayman Chemicals, Ann Arbor, MI) at concentrations of 1 to 100 μM at 37°C for 16 h unless otherwise indicated. Where indicated, cells were treated with peroxynitrite (100 μM; Upstate, Temecula, CA) or the peroxynitrite inhibitor [5,10,15,20-tetrakis (4-sulfonatophenyl)porphyrin iron (III), chloride] (FeTPPs) (20 μM; CalBiochem, San Diego, CA). Cells were then fixed in 4% paraformaldehyde (Sigma, 20 min), permeabilized in 0.1% Triton X-100 (Sigma, 20 min), blocked in 5% goat serum (Sigma, 1 h), stained with primary antibody (1:100, 1 h) and secondary antibodies (1:1,000, 1 h), and then imaged on an Olympus Fluoview1000 confocal microscope under an oil-immersion objective and standard filter sets as described (9). To ensure the specificity of immunodetection, slides were stained with secondary antibody alone or with primary antibody alone, thereby excluding the effects of autofluorescence and nonspecific binding of the secondary to the cells. In all cases, the lowest possible laser intensity was used for image detection. Images were analyzed, and surface expression of Cx43 was assessed by using Metamorph software version 6.1 (Universal Imaging) as follows: A differential interference contrast (DIC) image was obtained for each optical section, where the surface region of adjacent enterocytes was readily apparent. Regions of interest were then drawn at the cell surface. The frequency with which the immunostaining that was obtained under the fluorescent filter set occurred at the cell surface was recorded and quantified as a percentage of the total cells in each field. The process was repeated by two blinded observers examining over 100 confluent fields per treatment condition. For immunohistochemistry, a section of terminal ileum was harvested and immunostained for leukocytes by using antibodies against CD45 and actin by using rhodamine phalloidin (Molecular Probes, Eugene, OR) along with the nuclear marker 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) as described (6).

To measure F-actin polymerization, IEC-6 cells were cocultured with media, control macrophages, or activated macrophages and after 12 h were immunostained with rhodamine phalloidin (Molecular Probes), which detects the proportion of F-actin, then examined, and quantified for the rhodamine signal intensity by confocal microscopy.

Harvest of primary macrophages. Male iNOS−/− and C57BL/6 wild-type littermates (Jackson Laboratory, Bar Harbor, ME) at 8–12 wk of age were housed and treated in accordance with the Children’s Hospital of Pittsburgh Animal Care Guidelines and the Institutional Review Board of the University of Pittsburgh (protocol 08-05). Macrophages were harvested by peritoneal lavage as described (21). Briefly, immediately after euthanasia, the skin of the abdomen was cleansed with 70% ethanol, and an incision was made in the lower abdomen through the skin, leaving the peritoneum intact. Animals were then injected intraperitoneally with 10 ml ice-cold PBS, followed by gentle massage to allow for the distribution of the lavage fluid throughout the peritoneal cavity. The PBS lavage fluid was then removed using a 10-ml syringe attached to a 21-gauge needle, and...
cells were washed three times in DMEM and 10% FBS. Macrophages were identified by adherence to glass coverslips (1 h, 37°C) and morphology characteristics under light microscopy.

**Determination of enterocyte migration.** IEC-6 enterocytes were plated at 90–100% confluence overnight in the bottom well of a six-well plate (Corning) and then serum starved for an additional 12 h. In parallel, J774 macrophages were plated on a 0.4-μm pore size Transwell insert (Corning) overnight in a separate six-well plate (Corning). Prior to combining the macrophages and enterocytes, J774 macrophages were stimulated with LPS (10 ng/ml) and IFN (100 U/ml) for 6 h in the presence or absence of the iNOS inhibitor L-NIL (10 μM) for 1 h and then washed with copious amounts of PBS to remove residual LPS or IFN from the media. To initiate enterocyte migration, IEC-6 cells were scraped by using a cell scraper as described (43). The insert containing the macrophages was then combined with the enterocytes, and coculture was continued for an additional 24 h in a 37°C in a humidified incubator with 5% CO₂.

In parallel experiments, IEC-6 cells were pretreated with the NO donors SNAP (100 μM) or detaNONOate (100 μM) or the gap junction inhibitor oleamide (10 μM), and cells were allowed to migrate as above. In parallel experiments, cells were treated with peroxynitrite (100 μM) or the peroxynitrite inhibitor FeTPPs (CalBiochem, 20 μM).

Where indicated, SNAP (100 μM) was added to cells that were already migrating, either 1, 4, or 6 h after the initiation of migration. A calibration scale was obtained, and the migration rate was determined by measuring the mean distance traveled by 15 cells per field over the course of the experiment. Measurements were obtained from cells that were selected both at the leading edge and several rows back as described (9, 37) and quantified by using Metamorph software version 6.1 (Universal Imaging, Downingtown, PA).

**Quantification of gap junction communication in enterocyte monolayers.** Gap junction communication was assessed using the technique of single cell microinjection, which allows for the detection of the
extent to which the 0.4-kDa fluorescent gap junction tracer Lucifer yellow passes from an injected cell to adjacent cells through gap junctions. IEC-6 cells were grown to ~75% confluence on Labtek cell chambers (Nalge Nunc International, Naperville, IL) in the presence or absence of the NO donor SNAP (10–100 μM) or the gap junction inhibitor oleamide (10 μM), then bathed in injection buffer in mM (130 NaCl, 4 KCl, 1.8 CaCl₂, 0.56 MgCl₂, 10 glucose, 1.2 Na₂HPO₄, and 14.3 HEPES, pH 7.4), and transferred to the stage of an inverted microscope (Nikon Diaphot TMD). Individual enterocytes were then loaded onto the stage of an IX10 Olympus microscope and visualized with a 60× oil objective and then microinjected by using an InjectMan N2 Femtotip injector with which the fluorescent tracers were loaded into individual Femtotips II (Eppendorf, Hamburg, Germany). Each cell was injected for 0.2 s at an injection pressure of 30 hpa with both the gap junction tracer 0.4 kDa Lucifer yellow (25 mg/ml) and the 70 kDa gap junction impermeant fluorescent compound Texas Red Dextran (Molecular Probes, 10 mg/ml), which serves as a control for nonspecific dye passage. Enterocytes were injected both at the edge and within the culture, and cell-to-cell communication was determined. Fluorescent emission was quantified by using MetaMorph 6.1 (Universal Imaging) under standard filter sets. Cells were imaged prior to microinjection to assess for the presence of autofluorescence, which was subtracted from further analysis. The extent of dye coupling was determined by examining the level of transfer of Lucifer yellow to increasing tiers of neighboring cells. In all experiments, dye coupling was tested in a minimum of 10 microinjected cells (34).

Statistics. All experiments were repeated at least three times. All immunoblots were repeated in triplicate. For immunocytochemical microscopy, all experiments were repeated in triplicate, and over 100 fields were examined per experiment, in which each field was over 80% confluent. Comparisons were made by Student’s t-test and ANOVA where appropriate. Significance was accepted for *P* < 0.05.

RESULTS

Activated macrophages inhibit the phosphorylation of the gap junction protein Cx43 in enterocytes in a NO-dependent manner. The present studies were designed to test the hypothesis that activated macrophages could modify the gap junctions of adjacent enterocytes and that such effects could occur in a NO-dependent manner. The proximity of macrophages to enterocytes within the intestine is demonstrated in Fig. 1A. Given the inherent difficulties in assessing macrophage-enterocyte interactions in vivo, we turned to a reductionist approach to assess the effects, if any, of macrophages on enterocyte gap junctions. Specifically, we utilized the coculture system shown in Fig. 1, B and C, in which enterocytes were plated on the bottom of six-well plates and macrophages were plated onto Transwell inserts. Where indicated, macrophages were pretreated with a combination of LPS and IFN, resulting in a condition that will be described as “activated.” As is shown in Fig. 2A, treatment of macrophages with LPS and IFN led to the release of nitrite into the supernatant at levels significantly greater than groups of untreated macrophages. It is noteworthy that treatment of enterocytes with LPS and IFN in the absence of macrophages did not result in any significant increase in nitrite production, minimizing the possibility that enterocytes could release NO under these conditions. As expected, pretreatment of cells with the iNOS inhibitor 1-NIL (10 μM) significantly reduced the amount of nitrite release from macrophages compared with cells treated with LPS and IFN alone.

We next investigated the effects of activated macrophages on the state of gap junctions between adjacent enterocytes. We have recently demonstrated that Cx43, the most widely expressed gap junction constituent, plays a critical role in the regulation of enterocyte migration and that a reduction in the expression of phosphorylated Cx43 (pCx43) is associated with a significant reduction in the extent of gap junction communication that occurs between adjacent enterocytes (37). We therefore next assessed the effects of J774 cells that had been treated with LPS and IFN on the expression of pCx43 in adjacent IEC-6 enterocytes. As is shown in Fig. 2, B–E, coculture of enterocytes with activated macrophages resulted in a significant inhibition of pCx43 expression. Two lines of evidence prove that NO release from macrophages leads to the reduction in phospho-Cx43 expression in adjacent enterocytes. First, pretreatment of activated macrophages with the iNOS inhibitor 1-NIL led to a reversal of the inhibition of pCx43 expression (Fig. 2B). Second, coculture of enterocytes with macrophages that were obtained from iNOS⁻/⁻ mice did not reduce the expression of pCx43 in adjacent enterocytes, whereas coculture of enterocytes with macrophages obtained from iNOS⁺/⁺ mice demonstrated a significant reduction in the expression of enterocyte pCx43 (Fig. 2, C and D). Taken together, these findings demonstrate that macrophages inhibit gap junction phosphorylation of adjacent enterocytes and that this occurs in a NO-dependent manner.

In control experiments, we ruled out a generalized inhibition of cellular function from activated macrophages by using three approaches. In the first, we assessed the proliferative capacity of IEC-6 cells in the presence of media, unstimulated macrophages, and activated macrophages by using a colorimetric XTT proliferation assay and determined that the proliferation rate of the enterocytes was not affected by the addition of activated macrophages (data not shown). Secondly, there was no difference in the ability of enterocytes to exclude trypan blue in the presence or absence of activated macrophages, demonstrating structural integrity of the plasma membrane (data not shown). Thirdly, we determined there to be no difference in the rate of actin polymerization in IEC-6 cells in the presence or absence of activated macrophages (data not shown). Taken together, we feel that activated macrophages do not cause a generalized inhibition of cellular function and confer a more direct effect, in part, through the release of NO.

Activated macrophages cause an internalization of the gap junction protein Cx43 in adjacent enterocytes. Since the expression of pCx43 is directly linked with the extent of gap junction communication that occurs, the results of the previous experiments imply that activated macrophages may inhibit enterocyte gap junction function. Previous reports have also demonstrated that the internalization of Cx43 is associated with a decrease in gap junction communication (6, 35, 36), suggesting a link between internalization of gap junctions and reduced gap junction function. We therefore next investigated whether activated macrophages could alter the distribution of Cx43 in adjacent enterocytes. IEC-6 cells were plated to confluence in the presence or absence of activated macrophages, some of which had been pretreated with the NO inhibitor 1-NIL. As is shown in Fig. 3, A and D, the gap junction protein Cx43 is localized on the surface of enterocytes and is most highly expressed between adjacent cells. Importantly, coculture of enterocytes with activated macrophages resulted in a marked internalization of Cx43 within enterocytes to a site at which cell-to-cell communication would be expectedly inhibited (Fig. 3, B and D). Strikingly, treatment of macrophages with 1-NIL
prior to their activation led to a significant reversal in the redistribution of Cx43, restoring the gap junction protein again to the cell surface (Fig. 3, C and D). Treatment of cells with L-NIL alone had no effect (not shown). To directly evaluate whether NO release could impair Cx43 localization and phosphorylation in enterocytes, IEC-6 cells were plated to confluence and treated with the NO donors SNAP or detaNONOate. Whereas Cx43 was localized between untreated enterocytes and treated with the NO donors SNAP or detaNONOate. Where indicated, IEC-6 cells were left in media alone (media) or LPS and IFN together in IEC-6 cell lysates from IEC-6 cells that had been cocultured with J774 cells that were either untreated (Ctl Mø), treated with LPS and IFN (Møact.), or treated with LPS and IFN in the presence of L-Lysine ω-acetamidine hydrochloride (l-NIL) (Møact.+l-NIL). *P < 0.05 vs. control, representative of over 5 separate experiments. B: Western blot analysis showing the expression of phosphorylated Cx43 (pCx43) in IEC-6 cells that had been cocultured with J774 cells that were either untreated (Ctl Mø), treated with LPS and IFN (Møact.), or treated with LPS and IFN in the presence of l-NIL (Møact.+l-NIL), or treated with LPS and IFN in the presence of [5,10,15,20-tetakis (4-sulfonatophenyl)porphyrinato iron (III), chloride] (FeTPPs) (Møact.+FeTPPs), SNAP, or peroxynitrite. Blots were then stripped and reprobed with antibodies against actin to evaluate protein loading. The densitometry data revealing the ratio of expression of Cx43 to actin in the groups in the Western blot analysis above are shown, *P < 0.05 vs. the other 2 groups. Representative of at least 3 separate experiments. C: Western blot analysis showing the expression of pCx43 in IEC-6 cell lysates from IEC-6 cells that had been cocultured with peritoneal macrophages from either inducible nitric oxide synthase (iNOS) wild-type (iNOS+/+) or iNOS knockout (iNOS−/−) mice. Where indicated, IEC-6 cells were left in media alone (media). Blots were then stripped and reprobed with antibodies against actin to evaluate protein loading. D: densitometry data revealing the ratio of expression of pCx43 to actin in the groups. *P < 0.05 vs. the other 2 groups. Representative of at least 3 separate experiments.

Fig. 2. Activated macrophages cause a decrease in the expression of phospho-connexin43 (Cx43) in adjacent enterocytes in a nitric oxide (NO)-dependent manner. A: determination of supernatant nitrite concentration via the Griess reaction of IEC-6 cells that had been treated with either media alone (media) or LPS and IFN together (LPS+IFN); or IEC-6 cells in coculture with J774 that were either untreated (Ctl Mø), treated with LPS and IFN (Møact.), or treated with LPS and IFN in the presence of l-Lysine ω-acetamidine hydrochloride (l-NIL) (Møact.+l-NIL). *P < 0.05 vs. control, representative of over 5 separate experiments.
Fig. 3. Activated macrophages cause the internalization of the gap junction protein Cx43 in adjacent enterocytes in a NO-dependent manner. Representative immunofluorescent micrographs of Cx43 in IEC-6 cells that had been cocultured with J774 cells that were either untreated (Control Mφ, A), treated with LPS and IFN to activate the macrophages (activated Mφ, B), treated with LPS and IFN in the presence of l-NIL (activated Mφ+l-NIL, C), or treated with LPS and IFN in the presence of FeTPPs (activated Mφ+FeTPPs, D). Arrows point to the locations of Cx43 at gap junction plaques, either between or within enterocytes. E: relative percentage of IEC-6 cells in which Cx43 staining was localized to the cell surface as described in MATERIALS AND METHODS. Groups correspond to the experimental conditions in panels A–C, respectively. *P < 0.05, representative of 3 separate experiments. Size bar is 10 μm.

Fig. 4. NO donors cause the internalization of Cx43 and a decrease in the expression of pCx43 in IEC-6 enterocytes. A–C: representative immunofluorescent micrographs of Cx43 in IEC-6 cells that had been either untreated (control), treated with the NO donor SNAP (NO), or treated with peroxynitrite (peroxynitrite). D: relative percentage of IEC-6 cells in which Cx43 staining was localized to the cell surface as described in MATERIALS AND METHODS. Groups correspond to the experimental conditions in A–C, respectively. *P < 0.05, representative of 3 separate experiments. E: SDS-PAGE showing the expression of pCx43 in IEC-6 cells that had been treated with SNAP at the concentration indicated. Blots were then stripped and reprobed for F-actin. Graph indicates the densitometry data revealing the ratio of expression of pCx43 to actin. *P < 0.05 vs. untreated cells. Representative of at least 3 separate experiments. Size bar is 10 μm.
prevented by pretreatment of IEC-6 cells with the gap junction inhibitor oleamide (Fig. 5E), confirming that the dye was transferred via gap junctions. Importantly, treatment of confluent IEC-6 cells with the NO donor SNAP (Fig. 5E) significantly reduced the extent of gap junction communication that was detectable between adjacent enterocytes. These findings suggest the possibility that NO release by activated macrophages not only modifies the phosphorylation status and localization of gap junctions between enterocytes, but may also impair their functional status. Having recently demonstrated that intact gap junction communication between enterocytes is required for enterocyte migration (37), we next investigated whether macrophage activation could affect enterocyte migration, and if so, whether NO release could be involved.

**Activated macrophages inhibit the migration of adjacent enterocytes in a NO-dependent manner.** Healing from mucosal injury occurs through the migration of enterocytes from uninjured areas adjacent to the site of injury towards the wound (8, 42, 43). We have recently demonstrated that enterocyte migration requires intact gap junction communication between adjacent cells (37). Having now shown that activated macrophages may inhibit gap junction functional communication via the release of NO, we next sought to evaluate the effects of activated macrophages on the migration capacity of adjacent enterocytes. As is shown in Fig. 6, in the presence of control macrophages, adjacent enterocytes were able to partially close a wound by 24 h (Fig. 6, A, B, and G). By contrast, the presence of activated macrophages in association with enterocytes significantly reduced the ability of enterocytes to close the scraped...
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Fig. 6. Activated macrophages inhibit enterocyte migration in a NO-dependent manner. IEC-6 cells were plated onto glass coverslips on the bottom of 6-well plates and then cocultured with J774 cells that were either untreated (Control Mø, A and B), treated with LPS and IFN to activate the macrophages (activated Mø, C and D), or treated with LPS and IFN in the presence of L-NIL (activated Mø+L-NIL, E and F). IEC-6 cells were scraped and allowed to migrate over 24 h. The position of the leading edge of the cells at the beginning and end of the experiment is shown (dashed line). Size bar is 20 μm. G: in parallel, IEC-6 cells were cocultured with J774 cells that had been treated with LPS and IFN in the presence of FeTPPs (Mø+FeTPPs) or were treated with the NO donor SNAP (SNAP, 100 μM), the gap junction inhibitor oleamide (OLM), or peroxynitrite (perox), and the rate of migration was calculated. Shown also is the mean rate of migration of IEC-6 cells corresponding to the groups shown in A–E. Representative of at least 10 separate experiments, *P < 0.05 vs. control.

defect (Fig. 6, C, D, and G). Strikingly, pretreatment of activated macrophages with L-NIL prior to the onset of the coculture reversed the inhibitory effects of macrophages on enterocyte migration and restored the ability of enterocytes to close the defect (Fig. 6, E–G). Treatment of cells with L-NIL alone had no effect (not shown). It is important to note that treatment of enterocytes with either the gap junction inhibitor oleamide or the NO donor SNAP significantly impaired enterocyte migration (Fig. 6G), consistent with our previous findings (9, 37) and consistent with the important role for gap junctions in enterocyte migration. The addition of SNAP at various time points after the initiation of the scrape was found to lead to the arrest of enterocytes that were already migrating, as follows: mean ± SE μm/h: control, 7.4 ± 3; 1 h, 2.5 ± .8; 4 h, 3.2 ± 1; 6 h, 2.4 ± 1.1; P ≤ 0.05 vs. control by ANOVA. Importantly, the addition of peroxynitrite to cells did not affect the rate of enterocyte migration (Fig. 6G), and the pretreatment of activated macrophages with FeTPPs prior to the onset of the coculture did not reverse the inhibitory effects of macrophages on enterocyte migration, excluding the possibility that the effects of activated macrophages on enterocyte migration occurred via peroxynitrite. Taken together, these findings imply that enterocyte gap junctions as a target for macrophage-released NO in the inhibition of enterocyte migration, as may occur during diseases of intestinal inflammation.

DISCUSSION

Systemic sepsis may lead to intestinal epithelial dysfunction, resulting in mucosal injury and translocation of bacteria and macromolecules (17, 26). In response to mucosal injury, healing occurs through the process of enterocyte migration, which we have recently shown to require intact cell-to-cell communication via the gap junction protein Cx43 (37). Given the proximity of macrophages to enterocytes within the intestinal mucosa (46), we hypothesized that macrophage activation could affect the enterocyte barrier upon activation and sought to determine whether NO release was involved. We now demonstrate that activated macrophages cause a dramatic NO-dependent impairment in the phosphorylation and localization of the gap junction protein Cx43 in cocultured enterocytes, whereas exposure of enterocytes to NO leads to a profound inhibition in gap junction communication. We further demonstrate that activated macrophages prevent the ability of enterocytes to migrate into a scraped wound in a NO-dependent manner, consistent with a requirement for intact gap junction communication on enterocyte migration (37). Taken together, these findings suggest that macrophages inhibit enterocyte gap junctions via the release of NO, while impairing enterocyte migration. It is important to note that activated macrophages may not only interfere with healing, but may also initiate intestinal barrier injury in the first place through the release of NO and other proinflammatory molecules. Hence, activated macrophages within the intestine may be thought of as exerting a “double-hit” through the induction of tissue injury and the prevention of repair processes.

It is important to note that the concentration of SNAP utilized in the present experiments is within the physiological range that may be encountered in vivo. Specifically, data from a variety of studies from centers including our own indicate that levels of NO found in the intestine during conditions of inflammation are in the range of 10–150 μM (5, 16, 31, 49). To perform in vitro studies in which enterocytes are exposed to elevated concentrations of NO for a prolonged period of time (the migration assays occurred over 14 h) using the NO donor SNAP, we had to use concentrations up to 100 μM, given the half-life of this compound at 37°C (~5 h, for further details, see Ref. 4). It is noteworthy that a dose response was performed for key experiments in which the effects of NO on the
phosphorylation of Cx43 were determined by using concentrations between 0 and 100 μM (Fig. 4).

Although it is accepted that activated macrophages would be expected to release a variety of pro- and anti-inflammatory cytokines, several lines of evidence point to the importance of NO release in mediating the effects of activated macrophages on gap junctions. The effects of activated macrophages on gap junctions could be reversed after pretreatment of cells with the iNOS inhibitor L-NIL, and there were minimal effects of activated macrophages on Cx43 phosphorylation when macrophages were harvested from iNOS-deficient mice. Moreover, although it is possible that LPS and IFN may cause some release of NO from the enterocytes themselves, as has been reported by previous authors (18), we were not able to detect significant NO release from IEC-6 cells alone under these treatment conditions (Fig. 2A), providing further evidence for macrophage-mediated NO release in the effects of enterocyte gap junctions. Moreover, we did not detect a role for peroxynitrite in affecting either the phosphorylation or surface distribution of Cx43, excluding a role for this molecule. Despite this, we do acknowledge that other molecules released by activated macrophages may also affect gap junctions and, indeed, that effects of activated macrophages on the inhibition of enterocyte migration may occur independently of effects on enterocyte gap junctions. It is technically very difficult to definitively resolve this experimentally, given the absence of reliable methods to detect gap junction communication between enterocytes in real time during migration and the inability to “restore” the function of enterocyte gap junctions after exposure to activated macrophages. However, taken in aggregate, the observations that activated macrophages do, indeed, limit the ability of enterocyte migration to migrate into a scraped wound and the clear effects of NO release from activated macrophages on enterocyte gap junctions may provide useful insights into the complex cellular interactions that occur at the epithelial barrier during sepsis.

Although this is the first demonstration of an effect of activated macrophages on the capacity of adjacent enterocytes to undergo migration, previous authors have shed light upon the importance of enterocyte-immune interactions during inflammatory states. Takahashi-Iwanaga et al. (46) have used a combination of electron microscopy and fine section tissue imaging to demonstrate that lamina propria macrophages are specifically clustered beneath the enterocyte basement membrane, with their thick processes penetrating it, and that these macrophage protrusions participate in the maturation or aging of enterocytes as they migrate along the crypt-villus axis. Karrasch et al. (29) have recently shown that bacteria-induced experimental colitis involves the activation of Toll-like receptor (TLR)-induced NF-κB signaling derived mostly from mucosal immune cells, and, indeed, other authors have demonstrated an important role for lamina propria macrophages in the events that lead to bacterial translocation across the intestine (13, 48, 50). The present study builds upon these prior observations by others by showing that macrophages can affect the interconnectivity of enterocytes and that macrophages can exert effects on enterocyte migration. By identifying a critical role for NO in these processes, we now submit that the effects of macrophages on adjacent enterocytes may have a much broader role during inflammatory states than may have been appreciated.

What are the potential mechanisms by which NO could lead to a reduction in the expression of pCx43 and to a redistribution of surface Cx43 expression? One possibility is that NO may exert direct effects on the Cx43 molecule itself, resulting in nitration or nitrosation that may lead to changes in the phosphorylation or distribution of this protein, or, indeed, may inhibit the synthesis of the protein entirely. In support of this, NO-mediated posttranslational modifications have been shown to lead to a redistribution of dynamin in HEK- epithelial NOS (eNOS) cells (47), and to conformational changes of α-tubulin in A549 cells, an alveolar type II epithelial cell line (15). Alternatively, NO may alter the activation of other intracellular-signaling molecules that may themselves alter the expression or distribution of Cx43. In support of this, we have recently shown that NO may increase the activity of RhoA-GTPase (9), a family of signaling proteins that has been shown to modify the function of several membrane proteins including connexins (3, 40). Although this is the first study that has examined the role of NO on gap junctions between enterocytes, previous authors have demonstrated that NO may modulate gap junction proteins between a variety of other cells (27, 28, 41, 44, 45) through mechanisms that remain incompletely understood. We therefore now propose that through a combination of either direct effects on Cx43 itself or on the intracellular signaling pathways that modulate Cx43 expression or function, NO leads to changes in Cx43 in a manner that then inhibits gap junction communication between enterocytes.

The present experiments represent a novel departure from previously performed experiments in the field of intestinal inflammation by assessing the responsiveness of enterocytes to migrate while in association with activated macrophages. Although the experimental design is subject to the limitations of in vitro experiments, these studies adopt a reductionist approach to evaluate the migratory behavior of enterocytes under the multicellular conditions of the inflamed intestine. Given our recent finding that the proinflammatory environment of the inflamed intestine in neonatal necrotizing enterocolitis is associated with impaired expression and localization of gap junctions in vivo (37), it is tempting to speculate that inactivation of gap junctions by NO or other cytokines may underlie dysfunction in other organs that rely on coordinated cell-to-cell communication. In fact, impaired gap junction function could potentially precede histological evidence of tissue injury, providing a potential explanation as to why tissues obtained from patients dying from sepsis and multisystem organ failure may appear structurally intact, despite clinical evidence of profound functional impairment (25).

In summary, we have now shown that activated macrophages cause profound inhibitory effects on gap junction phosphorylation and localization in adjacent enterocytes and an inhibition of enterocyte migration in a NO-dependent manner. These findings shed light into the contribution of macrophage-enterocyte interactions in the pathogenesis of diseases of intestinal inflammation and identify enterocyte gap junctions as a potential target for the deleterious effects of proinflammatory agents in septic states.

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REFERENCES

41. Qureshi FG, Leaphart CL, Cetin S, Jun L, Grishin A, Watkins S, Ford HR, Hackam DJ. Increased expression and function of integrins in...


