Anti-inflammatory effects of epidermal growth factor on the immature human intestine

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Submitted 24 June 2011; accepted in final form 2 January 2012

Ménard D, Tremblay E, Ferretti E, Babakissa C, Perron N, Seidman EG, Levy E, Beaulieu JF. Anti-inflammatory effects of epidermal growth factor on the immature human intestine. Physiol Genomics 44: 268–280, 2012. First published January 3, 2012; doi:10.1152/physiolgenomics.00101.2011.—The inflammatory response of the preterm infants’ intestine underlines its inability to respond to hemodynamic stress, microbes, and nutrients. Recent evidence suggests that exogenous epidermal growth factor (EGF) exerts a therapeutic influence on neonatal enteropathies. However, the molecular mechanisms underlying the beneficial effects of EGF remain to be clarified. The purpose of this study was to evaluate the impact of EGF on the gene expression profiles of the developing human small and large intestine at midgestation in serum-free organ cultures using microarrays. The gene expression profiles of cultured human fetal ileal and colonic explants were investigated in the absence or presence of a physiological concentration of 50 ng/ml EGF for 48 h. Data were analyzed with the Ingenuity Pathway Analysis (IPA) software and confirmed by qPCR. We found a total of 6,474 differentially expressed genes in the two segments in response to EGF. IPA functional analysis revealed that in addition to differentially modulating distinct cellular, molecular, and physiological functions in the small and large intestine, EGF regulated the inflammatory response in both intestinal segments in a distinct manner. For instance, several intestinal-derived chemokines such as CCL2, CCL25, CXCL5, and CXCL10 were found to be differentially regulated by EGF in the immature ileum and colon. The findings showing the anti-inflammatory influence of exogenous EGF suggest a mechanistic basis for the beneficial effects of EGF on neonatal enteropathies. These results reinforce growing evidence that by midgestation, the human small intestine and colon rely on specific and distinct regulatory pathways.

Gene expression; microarrays; small intestine; colon; midgestation

Most of the functional development of the human intestine occurs during the first half of gestation (42, 46). In the small intestine, the villus structures start to develop according to a proximal-distal gradient between 8 and 11 wk of gestation so that at 18–20 wk, the morphology of the mucosa essentially resembles that of the newborn with a well-defined crypt-villus axis (36, 37). Several digestive enzymes associated with the enterocyte brush border such as the disaccharidases and peptides, as well as with intestinal lipid processing and lipoprotein synthesis, are present at midgestation at levels ranging from 70 to 100% of those of the adult intestine (31, 37, 41, 44, 62). The morphological development of the colon parallels that of the small intestine during the first two trimesters of gestation (37, 41). The formation of the villi begins between 11 and 14 wk of gestation under a distal-proximal gradient. Between 18 and 20 wk, the colonic mucosa is characterized by a well-defined crypt-villus architecture similar to that of the small intestine (37, 41). Small intestinal brush border digestive enzymes are also present in the fetal colon although their activities are lower than those of the small intestine (44, 62). Interestingly, in the human, although still poorly documented, the villi have been reported to remain present in the colon until ~30 wk of gestation at which time final maturation of the mucosa proceeds with the disappearance of the villus structures and loss of sucrase-isomaltase (SI) (56).

Despite structural and some functional similarities during the first two trimesters of gestation, recent gene expression profile analysis has identified fundamental differences between the two segments, most notably a general immaturity of the colon compared with the small intestine (75). Indeed, while overexpressed gene clusters in the ileum include genes involved with amino acid, vitamin, and mineral metabolism consistent with the absorptive function of the small intestine, predominant gene clusters expressed in the colon were mostly related to organ development and structural morphogenesis such as genes involved with cell cycle, cell death, and cell signaling. Taken together, these observations point out that, even at midgestation, these two intestinal segments are very different, both functionally and in terms of relative maturity, and thus should be considered as two distinct organs.

In recent years, medical advances have given rise to an increased survival of premature babies, but the relative immaturity of the intestinal mucosa (16, 26) has been associated with an inappropriate response to the early presence of nutrients and microflora, leading to severe pathological complications such as necrotizing enterocolitis, pneumatosis colli, and other intestinal distresses (12, 22, 27, 29, 30, 49, 51, 59, 73, 81). Potential strategies to overcome the onset of these pathologies include various treatments (51) such as antenatal administration of glucocorticoids (50) and enteral arginine supplementation (2, 17). Treatment with epidermal growth factor (EGF) is also of great interest as a potential strategy for the prevention of neonatal enteropathies (14, 19, 48, 52). Indeed, exogenous EGF has been proposed as a therapeutic agent to promote the healing of the gastrointestinal tract in pediatric patients (18, 48,
Enteral administration of EGF has been reported to reduce the incidence of necrotizing enterocolitis in a neonatal rat model (63). The mechanisms underlying the EGF-mediated protective effect in experimental animal models could be related to a reduction of epithelial cell apoptosis (15), a decrease in intestinal permeability (14), angiogenesis (78), and/or a decrease in autophagy (33). The mechanisms by which EGF promotes mucosal healing in the developing human gut are much less characterized (64, 69). By using organ culture of the human fetal intestine maintained under well-defined conditions, our group showed that intestinal functions are closely regulated by hormones and growth factors (36, 37, 41, 42). Interestingly, EGF was shown to downregulate epithelial cell proliferation in all segments but had distinct effects on the expression of brush border enzymes such as SI in the small intestine and colon (36, 40, 43).

In the present study, we exploited our serum-free organ culture technique (38, 39) and microarray analyses conjugated to the use of Ingenuity Pathways Analysis (Ingenuity Systems, http://www.ingenuity.com) to investigate the specific intestinal effects of EGF on the overall physiology of both the small and large intestine at midgestation. Our results showing that the inflammation-related gene families are among the major targets of physiological concentrations of EGF suggest a mechanistic basis for the beneficial effects of EGF in intestinal inflammatory diseases and also establish a segment-dependent effect of EGF on the inflammatory response suggesting that the location of the intestinal injured area needs to be taken into account.

MATERIALS AND METHODS

**Tissues.** Small intestinal (ileum) and large bowel (colon) tissues were obtained from cadavers of fetuses ranging from 16 to 20 wk (67) following legal or therapeutic pregnancy termination with informed patient consent. No tissues were collected from cases associated with known fetal abnormalities or intrauterine fetal demise. Studies were approved by the Institutional Review Committee for the use of human material from the Centre Hospitalier Universitaire de Sherbrooke/Faculté de Médecine et des Sciences de la Santé.

For the initial study, a first set of four specimens of small intestine (ileum) and three specimens of large intestine (colon) obtained from four fetuses ranging from 16 to 20 wk of age (postfertilization) were used. For the second and definitive study, another set of four specimens of small intestine (ileum) and four specimens of large intestine (colon) obtained from seven fetuses ranging from 17 to 19 wk of age were used.

**Serum-free organ culture.** Small and large intestinal tissues were prepared as previously described (38, 39). Briefly, gut mucosa was cut into 5 × 5-mm² explants and maintained in organ culture dishes (Falcon Plastics, Los Angeles, CA) at the interface of a 95% air-5% CO₂ gas mixture and culture medium for up to 2 days (37°C). For each intestinal tissue, four culture dishes were used, two for each experimental condition (untreated and EGF-treated). Human recombinant EGF (Collaborative Biomedicals, Bedford, MA) was added at a concentration of 50 ng/ml as done previously (40, 43). Explants were maintained in culture for 2 days.

**RNA extraction.** RNA was extracted with TRIzol (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s protocol and stored at −80°C. For the first set of samples (4 control ileums, 4 EGF-treated ileums, 3 control colons, 3 EGF-treated colons), quality of RNA was verified on agarose gel and by spectrophotometric assay. Poly(A) RNA was amplified using the TargetAmp 1-Round aRNA Amplification Kit (Epigenome Biotechnologies, Madison, WI). For the second set of samples (4 control ileums, 4 EGF-treated ileums, 4 control colons, 4 EGF-treated colons), quality was also confirmed by determining RNA integrity values >7.0.

**Probe preparation, screening, and data analysis for cDNA microarrays (set 1).** Probes were prepared for the 14 samples (4 control ileums, 4 EGF-treated ileums, 3 control colons, 3 EGF-treated colons) as previously described (75). Briefly, first-strand cDNA synthesis from 1 μg of aRNA was primed with 3 μg random hexamers (Invitrogen) by heating at 70°C for 10 min, snap-cooling on ice for 30 s, and incubating at room temperature (RT) for an additional 5–10 min. Reverse transcription was performed in the presence of 500 μM dATP, dCTP, and dGTP, 300 μM 5-aminoallyl-dUTP (Sigma) and 200 μM dTTP, 1 X first-strand buffer, 10 mM dithiothreitol, and 400 U Superscript II (Invitrogen) in a volume of 40 μl at 42°C for 3 h to overnight. cDNA was purified on QIAquick columns (Qiagen) according to the manufacturer’s directions. For all microarray experiments, the reference pool (63) was labeled with Cy3 dye, while test samples were labeled with Cy5 dye. Coupling reactions were quenched by the addition of 35 μl of 0.1 M sodium acetate, pH 5.2, and unincorporated dye was removed using QIAquick columns. The labeling efficiency was determined by analyzing the whole undiluted sample in a spectrophotometer using a 50 μl microcuvette (Beckman).

cDNA microarrays were obtained from the University Health Network (UHN) of Toronto, Ontario (Canada), and analyzed as previously described (74, 75). Slides were prehybridized in 0.1% BSA, 5 × SSC, 0.1% SDS for 45 min, washed by dipping in MilliQ water twice, 2-propanol once, and air-dried. Fluorescent cDNA probes were lyophilized and resuspended in 30 μl of hybridization buffer (50% formamide, 5 × SSC, 0.1% SDS). To the combined Cy3 and Cy5 samples, 20 μg Cot1 DNA, and 20 μg poly(A)⁺ DNA were added, and the samples were denatured at 95°C for 5 min, followed by snap-cooling on ice for 1 min. Room-temperature probes were applied to a prehybridized array, covered with another slide rather than a glass coverslip, and placed in a humidified hybridization chamber (Corning). Hybridizations were carried out at 42°C for 16–20 h, followed by 5 min washings in: 1 × SSC, 0.2% SDS at 42°C, 0.1 × SSC, 0.2% SDS at RT, and 0.1 × SSC at RT, twice. Arrays were scanned using a ScanArray Express dual-color confocal laser scanner (Perkin Elmer). Data were collected in Cy3 and Cy5 channels and stored as paired TIFF images. Spots were identified and local background subtracted using the TIGR Spotfinder 3.1.1 software (58). A quality control filter was used to remove questionable array features. Two criteria for spot rejection were a spot shape that deviated from a circle and a low signal-to-noise ratio. Hybridization intensity data were normalized using iterative mean-log(ratio)-centering (data range for mean centering ± 3 SD) and Lowess smoothing (procedure was set to 33%) using the native Java function of the TIGR MIDAS 2.19 software (Microarray Data Analysis System) (58) [data are accessible through Gene Expression Omnibus (GEO) and are all MIAME compliant]. Statistical significance was assessed by an unpaired t-test (P < 0.05), and hierarchical clustering analysis was performed using the TMEV 4.2 software (TIGR_MultiExperiment Viewer) (58). All software is available at The J. Craig Venter Institute website, http://www.jcvi.org/.

**Microarray screening and data analysis (set 2).** Probes for microarray analysis generated from RNA isolated from each experimental condition (16 samples) of cultured explants from both the small and large intestines (4 control ileums, 4 EGF-treated ileums, 4 control colons, 4 EGF-treated colons) were prepared at the microarray platform of the UHN Microarray Centre, University Health Network (Toronto, ON, Canada). Two Illumina whole genome Human HT-12 v4 expression beadchips (12 samples per beadchip) were screened and analyzed via the UHN Microarray Centre (data are accessible through GEO and are all MIAME compliant). To test for statistically significant changes in signal intensity (P values of <0.05), compiled data were screened using TMEV 4.2.

IPA. Functional analyses were performed with the use of IPA 8.8 software (Ingenuity Systems, http://www.ingenuity.com). The refer-
ence lists containing differentially expressed genes with gene identifiers and corresponding expression values were uploaded into the IPA application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Knowledge Base. IPA allows filtering to consider only functions and interactions in protein networks and/or pathways that are known for the defined species and tissue or cell line range. The stringent filter set for human and relaxed filter for tissues and cell lines were used for the core analyses. Fisher’s exact test was used to calculate a P value determining the probability that each biological function assigned to that data set would be due to chance alone.

Data validation by qPCR. The same 16 samples used for second set of microarray analyses were also used individually for qPCR confirmation, and all reactions were performed in duplicate. All reactions were performed in an Mx3000P real-time PCR system (Stratagene, Cedar Creek, TX) starting with 10 min of Taq activation at 95°C, followed by 40 cycles of melting (95°C, 30 s), primer annealing at the temperature appropriate for each primer (55–60°C, 45 s), and extension (72°C, 45 s) ending with a melting curve analysis to validate the specificity of the PCR products. Fluorescence data were acquired after each annealing step. Amplification efficiencies ranged from 91% to 105%. The Brilliant II SYBRGreen QRT-PCR Master Mix (Stratagene) was mixed with the appropriate primers and high-quality sterile water. The genes investigated were BMP4, CCL2, CCL14, CCL25, CEACAM1, CXC5, CCL10, DCC, DPP4, DUOX2, GSTA2, ITG2A, ITGAE, LCT, SI, STAT1, TM4SF4, TFF1, and Z02. Primers (Table 1) were generated using the primer formation software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with attention given to avoiding primer-dimer formation by stringent use of the maximum 3’ self-complementarity function of the Primer3 program.

Differences in gene expression were evaluated by comparing untreated vs. EGF-treated samples for a given intestinal segment using the equation R = (Etarget vs. EGF-treated samples for a given intestinal segment using the equation R = (Etarget)/(Ereference)/H9004. The reference gene used was the ribosomal protein S3A (RPS3A) (21).

Effects of EGF on gene expression profiles and functional networks in midgestation human small and large intestine. Explants from ileum and colon were cultured in the presence or absence of 50 ng/ml EGF for 48 h. Gene expression profiles were first analyzed in four ileums and three colons using cDNA microarrays containing 19,200 cDNA clones. We found 8,610 and 9,107 genes to be present in all tested samples for the ileum and colon, respectively. Unpaired t-test (P < 0.05) was applied to the ileum and colon data sets. We found 2,478 and 1,802 genes to be differentially expressed in response to EGF in the ileum and colon, respectively (Supplementary Tables S1 and S2).1 The original data have been deposited in the National Center for Biotechnology Information’s GEO and are accessible through GEO Series accession number GSE18367.

Differentially expressed genes in the ileum and colon in response to EGF treatment were subjected to IPA functional analysis. For each segment, a comparative analysis of the cellular, molecular, and physiological functions consisting of clusters of several individual genes was performed and listed according to statistical significance in the variation of the expression of genes in each category. Supplementary Tables S3 (ileum) and S4 (colon) list 78 categories that were sorted according to their statistical significance as the negative loga-

Table 1. Primers used in this study

<table>
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<tr>
<th>Gene Symbol</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Accession No.</th>
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<td>CEACAM1</td>
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<td>5'-GAAGAAAGCAAGATGGAAGA-3'</td>
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<td>CXC10</td>
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<td>CXC25</td>
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<td>DUOX2</td>
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<td>5'-CAGGGGCGTTCTCTGTTAGGA-3'</td>
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<td>NM_004817</td>
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1 The online version of this article contains supplemental material.
rithm of \( P \) values calculated by IPA. Plotting the negative logarithm of \( P \) values calculated by IPA for each of the functional categories found in the ileum against the negative logarithm of \( P \) values of the corresponding categories found in the colon allows visualization of those functions that are more relevant to each segment (Fig. 1A). More than 80% (63 out of 78 functions) of the significant categories identified in the ileum were shared with the colon. These functions included various biological processes known to be affected by EGF such as “cellular growth and proliferation,” “cell cycle,” “tissue development,” and “cell death” (Fig. 1A; see Supplementary Tables S3 and S4 for a complete list of functions and genes included). Nevertheless, several important metabolic functions were represented at significant levels exclusively in the ileum (13/78) such as “cell-mediated immune response,” “vitamin and mineral metabolism,” and “endocrine system development and function” (Fig. 1A), reflecting the more advanced maturity of the ileal mucosa as found earlier (75). Only two functions (2/78) were exclusively modulated by EGF in the colon including “lymphoid tissue structure and development.” Surprisingly, we observed that functional categories such as “inflammatory response,” “immune cell trafficking,” “inflammatory diseases,” and “gastrointestinal diseases” were significantly modulated in both segments (Fig. 1A), suggesting that, as early as midgestation, EGF could modulate an inflammatory response in the gut.

To further investigate the modulatory influence of EGF on inflammatory response in the midgestation intestine, a second set of explants obtained from four ileums and four colons were cultured in the presence of 50 ng/ml of EGF for 48 h. Gene expression profiles were then determined using Illumina whole genome expression beadchip microarrays providing coverage for >47,000 transcripts for each of the 16 samples. Statistical analyses revealed that 3,706 and 3,248 genes were found to be differentially expressed by EGF in the ileum and the colon, respectively, compared with controls (see Supplementary Tables S5 and S6 for gene lists). The original data have been deposited in the National Center for Biotechnology Information’s GEO and are accessible through GEO Series accession number GSE33806. As above, differentially expressed genes in response to EGF treatment in the ileum and colon were independently subjected to IPA functional analysis, and for each segment, a comparative analysis of the cellular, molecular, and physiological functions consisting of clusters of several individual genes was performed and listed according to statistical significance in the variation of the expression of genes in each category. Supplementary Table S7 (ileum) and Supplementary Table S8 (colon) list 68 and 74 categories, respectively, which were sorted according to their statistical significance as the negative logarithm of \( P \) values calculated by IPA. Plotting the negative logarithm of \( P \) values calculated by IPA for each of the functional categories found in the ileum against the negative logarithm of \( P \) values of the corresponding categories found in the colon allows visualization of those functions that are more relevant to each segment (Fig. 1B). More than 85% (63 out of 74 functions) of the significant categories identified in the ileum were shared with the colon. Interestingly, this IPA revealed the same trends as those from the IPA of the data generated with the cDNA microarrays (Fig. 1A). These functions still included various biological processes known to be affected by EGF such as “cellular growth and proliferation,” “cell cycle,” “tissue development,” and “cell death” (Fig. 1B) as well as the inflammatory-related functions such as “gastrointestinal diseases,” “immune cell trafficking,” “inflammatory diseases,” and “inflammatory response” were significantly modulated in both segments (Fig. 1B), confirming that, by midgestation, EGF can modulate an inflammatory response in the gut. The genes modulated by EGF for each of the four inflammation-related functions in the ileum and colon are listed in Table 2 and Table 3, respectively. As summarized in Table 4, a comparable number of genes from these categories were upregulated in both segments. However, ~70% of all differentially expressed genes in the colon forming these clusters were found to be downregulated by EGF compared with only 33% for the ileum, suggesting that EGF could induce a more important repressive effect on the inflammatory response in the colon than the small intestine. Furthermore, <7% of the inflammation-related genes modulated in either direction by EGF were common to both segments (Fig. 2). Taken together, these data confirmed that the inflammatory response can be modulated by EGF and appears to be regionally regulated in the gut.

**Regulation of inflammatory functions by EGF.** To further investigate the effect of EGF on intestinal inflammation, we selected genes from these inflammatory functional categories for qPCR confirmation. As illustrated in Fig. 3, we observed a segment-specific response for several genes, namely the pro-inflammatory chemokines CCL2 and CXCL10, which were exclusively downregulated in the colon, while the expression of CXCL5 was upregulated specifically in the ileum. The expression of other inflammation-related genes such as CCL25, TFF1, and CEACAM1 was similarly modulated by EGF in both segments (Fig. 3).

**Regulation of enterocytic differentiation markers by EGF.** Using SI and lactase (LCT) as intestinal organ culture references, we also observed that EGF differentially affected the levels of mRNA expression of SI and LCT in opposing directions in the developing small intestine but not in the colon (Fig. 4). These results corroborate those previously reported by our group at an earlier gestational age (11–14 wk) at the protein level (40, 43), indicating that EGF has a similar effect at different developmental time-frames along the human intestine.

**Validation of the microarray data.** To validate the microarray data, expression levels determined for a number of genes on the microarray were compared with those obtained with the same samples by qPCR. In addition to the eight genes used to estimate the inflammation- and differentiation-related effects of EGF in both ileum and colon (Table 5), another set of 12 genes was analyzed in either ileum or colon culture samples by qPCR (Table 6). The differentially expressed genes examined under each condition showed similar levels of change in expression which were not statistically different from that found in the array data as determined by a Pearson correlation test.

**Immunofluorescence.** To investigate the anti-inflammatory response elicited by EGF at the tissue level, the expression of two genes from the inflammatory functional categories modulated by EGF was investigated by indirect immunofluorescence in either ileum or colon explants cultured in the presence of EGF for 48 h (Fig. 5). In the ileum, positive staining for CXCL5 was exclusively found at the epithelial level in the
Positive staining for the proliferating antigen Ki67 in the colonic epithelium (Fig. 5F) confirmed the excellent viability of the tissue after 48 h of culture (3, 4).

**DISCUSSION**

Recently, the combined use of microarray analysis and bioinformatic software such as IPA has allowed our group to provide new insight into the morphological and functional development of the human gastrointestinal tract (75). More

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**Table 2. Inflammation-related cell, molecular, and physiological functions modulated by EGF in the small intestine at midgestation**

<table>
<thead>
<tr>
<th>Functions</th>
<th>Genes Upregulated</th>
<th>Genes Downregulated</th>
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<tr>
<td><strong>Gastrointestinal diseases</strong></td>
<td>ACSL5, ADORA2A, AKAP13, ANGPT2, ANTXR1, ANXA2, AREG/AREGB, ARG2, ATP2A2, ATP4B, BCR, BGN, BMP2, CACYBP, CAPG, CAPS10, CASP4, CASP9, CKD7, CDKN1A, <strong>CEACAM1</strong> (includes others), CES1, CHRNA1, CHST5, CLDN3, CLDN7, COL18A1, CTNNAA1, CTNNB1, DGKA, DCL1, DLG2, DSG2, DSTN, DUSP5, DUSP6, EFNB1, EGF, EGF2C2, EIF4A2, EIF4E, ELANE, EMX2, EPCAM, EPHA2, ERRF1, ESR, F3, FAS, FBXW7, FOXP2, FRMD3, FST, FUT4, ... S100A6, S100P, SFPI9, SPARG5, <strong>SERPINB5</strong>, SERPINB1, SERPIN2, SERT, S1F1, S1G1, S1G2, SLC16A3, SLC16A4, SLC2A3, SLC7A5, SMAD3, SOD2, SPINK1, SPON2, SPPL2A, SQLE, STARD13, SV420H1, SYCP1, TACSTD2, TAOK1, TCN1, TEAD4, TFF1, TFF3, TGBF4, TGBF5, TGBF6, TGBF7, TGBF8, TIP1, TIP2, TM4SF1, TM4SF4, TNC, TNFRSF6B, TUB3B, UBA1, UGT1A1, USP53, VPS28, VPS37A</td>
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<tr>
<td><strong>Inflammatory response</strong></td>
<td>ADORA2A, ANGPT2, CCL1, CD1D, CD2, <strong>CEACAM1</strong> (includes others), CXCL17, <strong>CXCL5</strong>, DP44, ELANE, FAS, HSP11, IL-16, IL-23A, ITGA3, ITGA5, ITGA6, <strong>MAGEA3/MAGEA6</strong>, MAEA, <strong>MMPI</strong> (includes EG:300339), MMP14, NCK1, PF4, PLAU, PPARG, RAC1, SELE, SERPIN1, TGBF2, TMBP1, TIP2, TNC, TNFRSF6B</td>
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<tr>
<td><strong>Immune cell trafficking</strong></td>
<td>ADORA2A, ANGPT2, CAST, CCL1, CCR6, CD151, CD2, <strong>CEACAM1</strong> (includes others), CXCL16, CXCL17, <strong>CXCL5</strong>, DP44, EDN2, EFNB1, EGF, F11R, F2RL1, FAS, GNAI3, HSP11, IL-16, IL-23A, ITGA2, ITGA3, ITGA5, ITGA6, ITGA7, ITGB1, MAGEA3/MAGEA6, MAPK1, MAP4, PLAU, PLAUR, PPARG, RAC1, RALGDS, S100A14, SELE, SERPINB3, SERPIN1, TAC1, TGBF2, TIP1, TIP2, TNC, TNFRSF6B</td>
<td></td>
</tr>
<tr>
<td><strong>Infectious diseases</strong></td>
<td><strong>ACHE</strong>, ADRB1K, AHNK, ALOX5, ARCN1, ASPN, ATP4B, BCL2L1, BGN, BMP2, CAPG, CASP4, CD55, CD59, CDK7, CFL1, CHM, DCL1, DNM1L1, DP44, DUSP1, EPHA2, F2RL1, F3, FAS, GRB7, HLA-DRB1 (includes EG:100332219), IDI1, IFI27, IL16, ITGA2, KRT16, LOR, NR4A2, P4HA2, PGM3, PTCHD1, RAB34, RUNX1, SERPINB3, SERPINB4, SERPINB5, SOD2, SPN, SPRR1B, TAC1, TAC1, TGF, TUB3B</td>
<td></td>
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</table>

EGF, epidermal growth factor. Genes appearing in boldface have been validated by qPCR.

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Fig. 1. Cellulair, molecular, and physiological functions modulated by epidermal growth factor (EGF) in the human small intestine and colon at midgestation. The negative logarithm of P values (Fisher’s test), calculated by Ingenuity Pathway Analysis (IPA), for each of the functional categories affected by EGF in the small intestine was plotted against the negative logarithm of P values of the corresponding categories in the colon. **A**: using sample set 1 and cDNA microarrays, IPA revealed that both intestinal segments share 80% of the 78 molecular and cellular functions, but some functional categories were found to be exclusively represented in each tissue (13/78 and 27/78 for the ileum and colon, respectively). **B**: using sample set 2 and Illumina whole genome expression beadchip microarrays, IPA revealed that both intestinal segments share 85% of the 74 molecular and cellular functions, but some functional categories were found to be exclusively represented in each tissue (57/4 and 11/74 for the ileum and colon, respectively). In both A and B, red and blue markers indicate inflammation-related functions and some developmentally related biological and cellular functions, respectively. Thresholds (dotted lines) denote the P = 0.05 [−Log (0.05) = 1.3], P = 0.001 levels [−Log (0.001) = 3] and P = 0.00001 levels [−Log (0.00001) = 5].

Physiol Genomics • doi:10.1152/physiolgenomics.00101.2011 • www.physiolgenomics.org
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specifically, this approach successfully identified key differences in the active functional networks between the small and large intestine in midgestation fetuses that need to be considered for the design of optimal treatments ensuring survival and growth of premature infants affected by devastating gastrointestinal diseases. Indeed, a significant finding from our previous clinical studies to exert positive effects in both the prevention and treatment of neonatal enteropathies (19, 48, 52, 64, 69).

Consistent with the apparent similar morphological and functional characteristics of the small and large intestine at midgestation (37, 41, 44, 62), the gene expression profiles compared with the small intestine raising the possibility that each segment may not respond equally to a given treatment. To test this hypothesis, we used EGF, which was previously shown in human clinical studies to exert positive effects in both the prevention and treatment of neonatal enteropathies (19, 48, 52, 64, 69).

Consistent with the apparent similar morphological and functional characteristics of the small and large intestine at midgestation (37, 41, 44, 62), the gene expression profiles established by IPA revealed that a large proportion (80%) of functions modulated by EGF were common to the two segments, whereas ~30% of functions were significantly modulated by EGF exclusively in the small intestine, and ~10% of functions were significantly modulated by EGF exclusively in the large intestine.

Table 3. Inflammation-related cell, molecular, and physiological functions modulated by EGF in the large intestine at midgestation

Table 4. Genes modulated by EGF in inflammation-related processes in the human intestine at midgestation

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while 11 of them (16%) including “lymphoid tissue structure and development” and “organ morphology” were identified as exclusive to the colon. From these data, it can be concluded that overall, EGF is a key modulator of gene expression related to a variety of gut functions at midgestation but in a context where the response differs considerably between small and large intestine. Such regional distinctions are in agreement with our previous studies (75).

The second finding from the IPA is the apparent effect of EGF on several inflammation-related functions such as “inflammatory response,” “inflammatory diseases,” “immunological diseases,” and “immune cell trafficking” in both segments. The result was confirmed using two independent sets of samples for both intestinal segments with distinct microarrays. Such a modulatory influence of EGF on the regulation of immune functions is consistent with previous observations showing a beneficial and or preventive effect of EGF on the intestinal mucosa of neonates (19, 48, 52). Indeed, while still incompletely understood, the mechanisms by which EGF may exert its effects could be through the modulation of chemokine expression as shown in the rat ileum (25). Another aspect of EGF’s action on the intestine is the regulation of maturation-related processes as seen in the developing human ileum and colon. Samples were normalized to RPS3A, and data are expressed as ratios of EGF treated over untreated segments, expressed on a Log2 scale. Values shown are the mean of 4 independent biological samples. *P < 0.05 vs. corresponding untreated control segments.

Table 5. Validation of inflammation- and differentiation-related gene expression profiles by qPCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Microarray</th>
<th>Real-time PCR</th>
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<tbody>
<tr>
<td></td>
<td><strong>EGF on ileum</strong></td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>0.84 ± 0.23</td>
<td>1.50 ± 0.57</td>
</tr>
<tr>
<td>CCL25</td>
<td>0.28 ± 0.08*</td>
<td>0.13 ± 0.04*</td>
</tr>
<tr>
<td>CXCL5</td>
<td>2.51 ± 0.58*</td>
<td>2.69 ± 0.35*</td>
</tr>
<tr>
<td>CXCL10</td>
<td>1.24 ± 0.62</td>
<td>1.00 ± 0.51</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>2.91 ± 0.44*</td>
<td>6.53 ± 1.63*</td>
</tr>
<tr>
<td>TFF1</td>
<td>2.67 ± 0.38*</td>
<td>3.93 ± 0.68*</td>
</tr>
<tr>
<td>LCT</td>
<td>1.46 ± 0.39</td>
<td>2.13 ± 0.19*</td>
</tr>
<tr>
<td>SI</td>
<td>0.49 ± 0.11*</td>
<td>0.46 ± 0.10*</td>
</tr>
<tr>
<td></td>
<td><strong>EGF on colon</strong></td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>0.56 ± 0.06*</td>
<td>0.48 ± 0.14*</td>
</tr>
<tr>
<td>CCL25</td>
<td>0.39 ± 0.16*</td>
<td>0.19 ± 0.12*</td>
</tr>
<tr>
<td>CXCL5</td>
<td>1.08 ± 0.22</td>
<td>0.98 ± 0.64</td>
</tr>
<tr>
<td>CXCL10</td>
<td>0.23 ± 0.06*</td>
<td>0.07 ± 0.05*</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>2.02 ± 0.53</td>
<td>2.86 ± 0.66</td>
</tr>
<tr>
<td>TFF1</td>
<td>3.37 ± 0.48*</td>
<td>2.65 ± 0.39*</td>
</tr>
<tr>
<td>LCT</td>
<td>1.33 ± 0.39</td>
<td>0.92 ± 0.30</td>
</tr>
<tr>
<td>SI</td>
<td>0.78 ± 0.13</td>
<td>1.21 ± 0.71</td>
</tr>
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</table>

Real-time quantitative PCR analysis of transcript levels of different genes in developing human intestine. Samples were normalized to RPS3A using the approach: \( R = \frac{(E_{target})^{C_{target}}}{(E_{reference})^{C_{reference}}} \). Microarray data are expressed as fold difference of expression (EGF/untreated). Values shown are the mean of 3 or 4 independent biological samples (*P < 0.05). Correlation analyses (Pearson correlation test) revealed that microarray data were positively correlated with qPCR data for ileum (\( P = 0.019, r = 0.88 \)) and colon (\( P = 0.01, r = 0.91 \)).
disclosed in our study is the major difference in both the number of genes modulated and in their patterns of expression between the small and large intestines. For instance, for the “inflammatory disease” cluster, 56 genes were found to be upregulated by EGF in the small intestine compared with 19 in the colon, while no genes were downregulated in the small intestine compared with 163 in the colon. A similar gene profile was also observed for the “gastrointestinal diseases” and “inflammatory response” categories. Moreover, in these inflammation-related functions, only a small proportion of the genes having their expression modulated by EGF were common to both the ileum and the colon further emphasizing the segment-specific modulating effect of EGF on the intestinal inflammatory response. To directly investigate this phenomenon, we analyzed the effect of EGF on the specific expression of individual genes associated with these functional categories: CCL2, CCL25, CXCL5, CXCL10, CEACAM1, and TFF1.

Proinflammatory chemokines CCL2 and CXCL10 were downregulated by EGF in the colon but were unchanged in the small intestine. Increases of CCL2 have been observed in mucosal tissues from patients with Crohn’s disease and ulcerative colitis (5, 34, 55) and also in experimental models of colitis (60, 70). Interestingly, it has been demonstrated in keratinocytes that EGFR appears to be strongly involved in the control of epithelial cell-triggered inflammation by downregulating the expression of CCL2 and CXCL10, which attracts diverse leukocyte subsets (32). A previous report showed that the expression of CXCL10 was elevated in inflammatory bowel diseases (66) and an overexpression of the CXCR3 axis components including CXCL10 was reported in childhood inflammatory bowel disease (61). Interestingly, CXCL10 can be expressed by epithelial cells suggesting that it may participate in the modulation of pathologic T cell-mediated mucosal inflammation (20). In a mouse model mimicking colitis, neutralization of CXCL10 protects from the development of acute colitis (71), suggesting a key role for this chemokine in the development of inflammatory bowel disease. The EGF-mediated downregulation of CCL2 and CXCL10 expression in the colon at midgestation identifies a new potential mechanism for the positive effects of EGF on mucosal healing in inflammatory intestinal diseases. Further studies should test these possibilities.

For CCL25, a strong downregulation of the expression by EGF was observed in both the small intestine and colon. CCL25 is an important chemokine predominantly expressed in the small intestine compared with the colon. Although the role of CCL25 still needs to be better defined in the human under both physiological and pathological conditions (76), the results from studies of animal models of IBS suggest that CCL25 and its receptor CCR9, which have roles in lymphocyte migration to the intestine, might contribute to chronic inflammation (79, 80). The five- to 10-fold downregulation of CCL25 by EGF in both intestinal segments pointed out another potential anti-inflammatory EGF target in the gut mucosa.

Interestingly, we found an increased expression of CEACAM1 by EGF in both intestinal segments. Our immunolocalization studies revealed that CEACAM1 was exclusively expressed by the epithelial monolayer, suggesting that observed increases are associated with this cell population. The inhibitory role of CEACAM1 in the regulation of mucosal inflammation has been raised in the last decade (47). Recently, a decrease of expression of CEACAM1 was observed in Crohn’s disease but not in ulcerative colitis (57), suggesting that a defect in CEACAM1 could play a role in the development of this disease. Moreover, phosphorylation of CEACAM1 by EGFR following stimulation by EGF has been demonstrated in the HT29 colorectal cancer cell line (1), supporting our observation of a connection between CEACAM1 and EGF in the human intestine. However, further studies will be needed to better define the role between EGF and CEACAM1 in the inflammatory response.

The significant upregulation of the proangiogenic chemokine CXCL5 (68) in response to EGF stimulation exclusively in the small intestine is another example of a tissue-specific EGF effect. Interestingly, it has been recently demonstrated in mice that EGF directly enhanced CXCL5 expression in the endothelial cells of villus capillaries to induce both new capillary growth and villus height during adaptation after small bowel resection (35). However, in the human midgestation small intestine, we observed that CXCL5 was exclusively located at the epithelial level in the bottom of the crypt. These findings corroborate those previously reported that demonstrated the same epithelial localization of CXCL5 in the human (28, 82). Whether the enhanced expression of CXCL5 observed in the human could be related to intestinal adaptation as observed in mice (35) remains to be elucidated. However, when one considers that EGF receptors were predominantly detected in the basolateral domain of epithelial cells in both the small and large fetal intestine (45, 54), it is conceivable that the epithelium may represent the main target of EGF as also suggested above for CEACAM1. This is consistent with the central role played by the epithelium in the mediation of the intestinal inflammatory response (16, 49).
Finally, we also found that TFF1 (trefoil factor family 1), a peptide playing an important role in protecting the gastrointestinal mucosa, was upregulated by EGF in both intestinal tissues. It has already been reported that systemic administration of TFF1 reduced inflammation in colitis and that the combination of EGF and TFF1 had a synergistic healing effect in a rat model of colitis (23). Our results indicate that the effect of EGF is not just restricted to modulation of proinflammatory...
molecules but also to regulation of the preservation of the integrity of the developing intestinal mucosa.

In conclusion, these results reinforce growing evidence that, at midgestation, the human small intestine and colon have specific and distinct regulatory pathways and that these differences extend to their response to growth factors. Indeed, previous work from our group showed that EGF was involved in the regulation of cell proliferation and differentiation in the fetal human small and large intestine (40, 43) as well as in the fetal rodent intestine (7, 8) even at earlier stages of development. The findings of the present study further show that inflammatory functions are among the main targets of EGF in the midgestation intestine, even when tested at physiological levels. As exemplified by the upregulation of the expression of genes linked with an anti-inflammatory/healing response (CEACAM1 and TTFT1) and a downregulation of other genes considered to be associated with proinflammatory conditions (CCL2, CXCL10 and CCL25), our data suggest that EGF exerts a net anti-inflammatory influence on the midgestation intestinal mucosa. Recent observations have brought to light that enterocyte immaturity could be involved in the excessive inflammatory response of the intestine in premature infants leading to necrotizing enterocolitis (49). There is also compelling evidence that abnormal EGF regulation in the preterm infant may contribute to the development of neonatal necrotizing enterocolitis (13). While EGF was shown to exert beneficial effects on the expression of a subset of interleukins in the ileum of a rat model of necrotizing enterocolitis (25), the present study is the first to demonstrate the direct capacity of EGF to exert anti-inflammatory effects on the human intestinal mucosa under defined conditions. In this context, the data presented herein suggest a mechanistic basis for better understanding the beneficial effects of EGF on mucosal healing in a variety of intestinal pathologies. Furthermore, these data establish that hereafter, the small intestine and colon need to be considered as distinct organs as early as midpregnancy both functionally and, more importantly, in response to therapeutic agents. These aspects need to be taken into account by clinicians since, as illustrated herein with EGF on the inflammatory response, the efficiency of specific therapies may depend on the location of the injured area.

ACKNOWLEDGMENTS

The authors thank Elizabeth Herring for reviewing the manuscript and Nuria Basora for suggestions. We thank Drs. C. Poulin and F. Jacot from the Département de santé communautaire du CHUS for excellent collaboration in preparing figures; E.T. and J.-F.B. drafted manuscript.

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