Expression and localization of pannexin-1 hemichannels in human colon in health and disease


*School of Medical Sciences, University of New South Wales, Sydney, New South Wales, Australia
†Sydney Colorectal Associates, St George Hospital, Sydney, New South Wales, Australia

Abstract

Background Pannexin-1 (Panx1) proteins can function as channels for adenosine triphosphate (ATP) release, but there have been limited studies investigating their potential role in the human intestine. The aim of this study was to characterize Panx1 expression and distribution in the human colon and its potential involvement in inflammatory bowel diseases (IBD).

Methods Human colon segments were dissected into mucosa and muscularis layers, and evaluated for Panx1 expression by real-time PCR and Western blotting. Immunohistochemistry was conducted to localize the cellular distribution of Panx1 in intact tissues.

Key Results In the colonic muscularis of ulcerative colitis (UC), Panx1 mRNA expression showed a 3.5-fold reduction compared with control (P = 0.0015), but no change was seen in UC mucosa. In contrast, down-regulation of Panx1 mRNA was observed in both muscularis and mucosa of Crohn’s disease (CD), showing a 2.7- and 1.8-fold reduction, respectively (P < 0.05). There was reduced Panx1 protein expression in CD muscularis, but no change in CD mucosa, UC muscularis, or UC mucosa. Pannexin-1 immunoreactivity was mainly localized to enteric ganglia, blood vessel endothelium, erythrocytes, epithelial cells, and goblet cells. Inflammatory bowel disease samples showed a similar overall pattern of Panx1 staining, but in UC myenteric ganglia, there was a significant reduction in Panx1 immunoreactivity. Significant Panx1 positive leukocyte infiltrations were seen at the sites of inflammation.

Conclusions & Inferences The presence of Panx1 in the colon and changes to its distribution in disease suggests that Panx1 channels may play an important role in mediating gut function and in IBD pathophysiology.

Keywords Crohn’s disease, human colon, pannexin-1, ulcerative colitis.

INTRODUCTION

Pannexins [Panxs] form cell membrane channels and have a similar tertiary structure to connexins—the gap junction proteins.¹ The Panx family consists of three members; Panxs 1–3.¹² Pannexins are often compared to connexins due to their topological similarities in structure, and as a result were first hypothesized to function as paired hemichannels allowing communication between cells.³ However, Panxs do not form docked junctions in vivo, instead behaving like unpaired connexin hemichannels.⁴,⁵ Pannexins act as conduits for adenosine triphosphate (ATP) release in response to physiological and pathological stimuli.⁶,⁷ The mechanism underlying Panx-mediated ATP release may involve increases in calcium release from the endoplasmic reticulum.⁷ Adenosine triphosphate released from Panx channels activates purinergic (P2Y) receptors, which cause inositol-3-phosphate formation, and an increase in intracellular calcium, promoting the further opening of Panx channels and propagation of a calcium wave through the tissue.⁷ There is also evidence that in macrophage cells, Panx1 and the purinergic P2X₇ receptor form a signaling complex and its activation leads to ‘large’ pore formation, ATP release, and paracrine activity.⁸ Pannexin-1 may also be involved in cell death when activation of the Panx1/P2X₇ complex is prolonged.⁹

Pannexin-1 channels are found associated with a number of cellular functions.⁴ The Panx1 protein is localized to the apical surface of tracheal epithelial cells where it plays an apparent role in maintaining
ATP levels at the mucosal surface. Pannexin-1 on erythrocytes regulates blood flow and local oxygen delivery. Pannexin-1 is implicated in immune function and T-cell activation via a P2X1 and P2X4 receptor-dependent calcium influx mechanism and is involved in the regulation of T-cell-mediated antigen presentation and other T-cell functional responses. Furthermore, Panx1 on macrophages has been shown to play a role in inflammation, where down-regulation of Panx1 was associated with the inhibition of interleukin 1β (IL-1β) expression when coupled with the P2X7 receptor.

There is little evidence for Panx channels in the intestine though one study showed expression of Panx-like proteins, innexins, in the gut of the nematode where they were important for gut motility. A recent study conducted in a mouse model of colitis showed that Panx1 is required for P2X7 receptor-mediated enteric neuron cell death in intestinal inflammation. As recent studies have shown a relationship of Panx1 expression with inflammation, we hypothesized that inflammatory bowel disease (IBD), i.e., ulcerative colitis (UC) and Crohn’s disease (CD), would be associated with altered Panx1 expression. Thus, the aim of this study was to assess Panx1 expression in colonic specimens from patients with UC and CD, in comparison with age-matched control colon. Altered Panx1 expression in diseased colon would be suggestive of their having a role in IBD pathophysiology.

MATERIALS AND METHODS

Patients and specimens

Sigmoid and ascending colon segments (~3 cm) were obtained from male and female patients undergoing total or partial colectomy for UC and CD (for detailed demographic and clinical information see Table 1). The edges of surgical specimens were dissected free of tenia coli, and the remaining tissue was separated into mucosal and muscle samples. Each muscle sample contained a thick layer of circular muscle, a thin layer of longitudinal muscle, and myenteric plexus, and hence was termed as muscularis externa (muscularis for short) in this study.

Colon specimens from carcinoma patients (age range 21–83 years, median 51 years, n = 28, 20 sigmoid and eight ascending) were used as control, and these samples were taken 10–20 cm away from the tumor. Control specimens with inflammation or macroscopic abnormality and those with obstruction, or from patients who had undergone radiation therapy or chemotherapy, were excluded.

For real-time PCR and Western blot studies, the colon specimens were dissected free of tenia coli, and the remaining tissue was separated into mucosal and muscle samples. Each muscle sample contained a thick layer of circular muscle, a thin layer of longitudinal muscle, and myenteric plexus, and hence was termed as muscularis externa (muscularis for short) in this study.

This project was approved by the Human Ethics Committees of the University of NSW and the St George Hospital.

Gene expression by real-time RT-PCR

The methods for tissue dissection, RNA extraction, and single strand cDNA (sscDNA) synthesis were described previously. Total RNA was extracted from muscularis and mucosal tissues using the Trizol method (Invitrogen, Life Technologies, Mulgrave Vic, Australia) followed by a DNase treatment (3 U at 37 °C for 20 min). Total RNA (2 µg) was used to synthesize sscDNA using a SuperScript™ III First-Strand Synthesis System (Invitrogen) and random hexamers (50 ng µL⁻¹) at 1 cycle of 25 °C for 10 min, 50 °C for 50 min, and 85 °C for 5 min. Newly synthesized sscDNA was treated with RNase H (2 U µL⁻¹) at 37 °C for 20 min to digest the RNA template. The concentration of sscDNA product was measured with a spectrophotometer and diluted to a working concentration of 500 ng µL⁻¹.

Real-time quantitative RT-PCR was carried out to determine the expression levels of mRNA for Panx1 and GAPDH (housekeeping gene; HKG) in UC and CD in comparison to controls, using KAPA SYBR FAST Universal qPCR kit (Kapa Biosystems, Woburn, MA, USA). Each PCR reaction was performed in a volume of 20 µL containing 10 µL of SYBR green master mix, 500 ng sscDNA, 10 pmol forward and reverses primers. The primer sequences are as follows: Panx1 FP: CTGTGGACAAGATGGTCACG and RP: CAGCAGGATGTAGGGGAAAA, GAPDH FP: ATGGGGAAG GTGAAGGTC and RP: GAGGTCAATGAGGGTGTAT.

In each RT-PCR assay, a designated calibrator RNA was used to allow inter-run comparisons. The PCR amplification conditions were 1 cycle at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 68 °C for 20 s. In the final step, the melting curve analysis was carried out during gradual temperature elevation from 60 to 95 °C. The PCR amplification efficiencies for Panx1 and GAPDH were 98% and 95%, respectively.

Table 1 Demographic and clinical information of disease specimens

<table>
<thead>
<tr>
<th>Demographic and clinical information of disease specimens</th>
<th>Ulcerative colitis (n = 11)</th>
<th>Crohn’s disease (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Males (9) and females (2)</td>
<td>Males (5) and Females (4)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Median 55, range 33–84</td>
<td>Median 40, range 26–77</td>
</tr>
<tr>
<td>Disease history</td>
<td>2–20 years</td>
<td>No history [2], 5–12 years [7]</td>
</tr>
<tr>
<td>Use of medications</td>
<td>Corticosteroids [10], 5-aminosalicylic acid [7], azathioprine [5]</td>
<td>No pretreatment [2], 5-aminosalicylic acid [5], 6-mercaptopurine [6], infliximab [3]</td>
</tr>
<tr>
<td>Disease localization</td>
<td>Pancolitis [5], left sided [4], right sided [2]</td>
<td>Terminal ileitis/right colon [4], pancolonic [4], anorectal [1]</td>
</tr>
<tr>
<td>Region of specimens studied</td>
<td>Sigmoid [7], ascending [4]</td>
<td>Sigmoid [1], ascending [8]</td>
</tr>
</tbody>
</table>

The numbers in brackets represent patient number.

© 2013 John Wiley & Sons Ltd
Western blot analysis

Protein was extracted from mucosa and muscularis samples (100 mg) using lysis buffer containing 1 mmol L⁻¹ EGTA, 10 mmol L⁻¹ Tris-HCl, and protease inhibitor cocktail tablets (Roche Applied Science, Castle Hill, NSW, Australia) and denatured at 95 °C for 8 min. Protein samples (40 μg) were loaded into 50 μL wells of 10% Mini-PROTEAN™ gels (Bio-Rad, Hercules, CA, USA) for electrophoresis at 200 V for 45 min. Proteins from gels were transferred to Polyvinylidene Fluoride membranes (Bio-Rad) by layering pads and filter papers to sandwich the gel and the membrane for 45 min at 90 V. The membrane was loaded onto blot holders of the SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA) and subjected to immunodetection according to the manufacturer’s protocol. Anti-Panx1 antibody (1 : 500 dilution, ab60098, Abcam, Waterloo, NSW, Australia) was incubated with the membrane for 20 min. Following washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1 : 1500, A4914; Sigma, Castle Hill, NSW, Australia) was incubated with the membrane for 20 min. Following washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1 : 1500, A4914; Sigma, Castle Hill, NSW, Australia) for 2 h. After incubating with avidin biotin complex (1 : 200, Vector Laboratories) for 1 h at room temperature, the slides were washed in tri-phosphate buffered saline (TBS-TX, 0.1 mol L⁻¹, pH = 7.6), for 3 x 10 min and incubated in 1 : 200 of biotinylated goat anti-chicken secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 2 h. After incubating with avidin-biotin complex (1 : 200, Vector Laboratories) for 1 h at room temperature, the slides were stained in DAB-nickel solution until the brown signal developed [Sigma], and counterstained with hematoxylin [Sigma] for 3 min. Each experiment included a negative control incubated with secondary antibody alone, and these negative controls consistently exhibited no immunoreactivity. Sections were scanned using the Aperio ScanScope XT digital scanning system (Aperio ImageScope, San Diego, CA, USA).

Anti-Panx1 antibody 4515 is raised against the carboxyl-terminal amino acids of the Panx1 protein sequence. We compared this antibody with another commercially available anti-Panx1 antibody (Abcam, ab124131, 1 : 500 dilution) in four of the human colon specimens pretreated with Target Retrieval Solution (Dako, Noble Park, VIC, Australia). Antibody ab124131 is raised against residues 150-250 of the human Panx1 sequence, corresponding to the region between intracellular loop 2 and extracellular loop 2. We found that the immunoreactivity generated by the two epitope-distinct antibodies appeared identical.

Data expression and analysis

For RT-PCR studies, a control muscle was used as an inter-run calibrator. The mRNA level was expressed as fold change, in which Panx1 gene (target gene) was normalized to GAPDH, the housekeeping gene [HKG], of the same sample, and then expressed relative to the calibrator Panx1 expression using the formula: fold change = 2^{-ΔΔCt}, where ΔΔCt = [Ct(target) - Ct(HKG)]sample - [Ct(target) - Ct(HKG)]calibrator. Region-, gender-, and age-matched controls were used. Data were presented as median and interquartile range (IQR) and Mann-Whitney nonparametric test were used for two group comparison using Graph Pad Prism Version 5.03 (San Diego, CA, USA) as data were not normally distributed. A P < 0.05 was considered to be statistically significant.

RESULTS

Expression levels of Panx1 mRNA in human colon

Real-time PCR was initially performed with RNA samples to determine the expression levels of Panx1 genes in control human colonic muscularis and mucosa. DNA gel electrophoresis showed that the expected PCR products for Panx1 (245 bp) mRNA were present in the colonic muscularis and mucosa, suggesting that the gene encoding Panx1 is expressed in the human colon. Quantitative real-time PCR was then performed to determine whether altered Panx1 gene expression occurs in IBD. The median level for Panx1 mRNA expression in control muscularis was 1.35 (IQR: 0.87–2.82) and in UC muscularis was 0.39 fold change (IQR: 0.19–0.70) relative to the calibrator, showing a 3.5-fold down-regulation of Panx1 mRNA in UC muscularis (Fig. 1A, P = 0.0015). Likewise, a significant reduction in Panx1 gene expression was observed in CD muscularis (2.7-fold, P = 0.039, Fig. 1B). There was no difference in Panx1 mRNA levels in UC mucosa compared with control (Fig. 1C). A small, but significant reduction in Panx1 expression was seen in CD mucosa (1.8-fold, P = 0.015, Fig. 1D).

Expression levels of Panx1 protein in human colon

The Panx1 protein band (Fig. 2A and D) was consistent with an ~45 kDa protein size as indicated by the manufacturer and a previous publication.© 2013 John Wiley & Sons Ltd
those of GAPDH (Fig. 2A). No change in expression level for Panx1 protein was observed in either UC muscularis or mucosa (Fig. 2B and C).

Pannexin-1 in CD muscularis (Fig. 2D) showed a 3.9-fold down-regulation of Panx1 compared with control \( (P = 0.0005, \text{Fig. 2E}) \). There were no statistically significant changes in Panx1 protein expression in CD mucosa compared with control mucosa (Fig. 2F).

**Immunohistochemical localization of Panx1 in human colon**

Pannexin-1 immunoreactivity [Panx1-ir] was present in all layers of the colon. In the colonic mucosa, Panx1-ir was localized to the cytoplasm and cell membranes of the epithelial and goblet cells [Fig. 3A and B]. Polymorphonuclear leukocytes, but not lymphocytes in lamina propria were positive for Panx1 labeling [0–6 cells per field under \( \times 40 \) magnification]. Some nerve fibers and small blood vessels in lamina propria showed strong Panx1 labeling (Fig. 3A and B). Pannexin-1-ir on muscularis mucosa was weak (Fig. 3B). In submucosa, the most prominent Panx1-ir labeling was seen on submucosal ganglia, the endothelial cells of blood vessels, erythrocytes and leukocytes (Fig. 4A–C). These findings were consistent across all specimens examined \( (n = 11) \). Weak Panx1-ir was also seen on the smooth muscle of blood vessels (Fig. 4B). In the muscularis externa layer, strong Panx1-ir signal was localized to varicose fibers within the myenteric plexus and to most,
but not all, nerve cell bodies (Table 2, Fig. 5A). Weak to moderate Panx1-ir was seen on the colonic smooth muscle of control specimens (Table 2, Fig. 5A and B). Compared with control mucosa, all UC specimens showed destruction of the colonic mucosal architecture, typically involving damage to the epithelial lining and crypts (Fig. 3C and D). Pannexin-1-labeled blood vessels were more prominent in UC mucosa due to the increased number of blood vessels, accompanied by infiltration of numerous Panxl-positive non-lymphoid leukocytes (Fig. 3C, Table 2). A marked increase in Panx1-negative lymphocytes was also seen in UC mucosa. The high population of these cells suggests that chronic immune activation is present in the affected areas of the colon. In some UC specimens, the thickness of muscularis mucosae was substantially increased (Fig. 3D), indicating tissue edema likely due to the inflammatory process. There was slightly reduced Panx1 signal in UC submucosal ganglia compared with control (Fig. 4D). The number of blood vessels in UC submucosa was significantly increased, many of which were sinusoidally distended, indicating tissue edema (Fig. 4E and F). Pannexin-1-ir showed no difference between UC and control smooth muscle (Fig. 5C and D). The most noticeable change was the marked reduction in Panx1-ir in UC myenteric ganglia (Fig. 5C).

The degree of damage of mucosal architecture varied in CD specimens with some appearing normal while others showed moderate to severe damage, which corresponded to the higher density of inflammatory cells in the lamina propria (Table 2). Figs 3E and F show a severe CD case. Substantial inflammatory cell infiltration was seen in the mucosal and submucosal regions, and the muscularis mucosae layer was completely destroyed (Fig. 3F). There was no obvious change in Panx1-ir density on CD submucosal ganglia and blood vessels (Fig. 4G–I), or on myenteric ganglia and smooth muscle (Fig. 5E and F), although the morphology of enteric neurons in CD appeared distorted (Figs 4G and 5E). A significant increase in serosal thickness, along with an increase in the number and size of blood vessels was present in the serosa of many CD samples (Fig. 5F), implying transmural inflammation, a key feature of CD.

DISCUSSION

A key finding of this study is that Panx1 is strongly and broadly expressed in the human colon and this
expression is altered in IBD patients. These data suggest that Panx1 channels may participate in a wide spectrum of biological functions in the colon and is consistent with a role for Panx1 in the pathophysiology of IBD.

Our data show that Panx1 mRNA and protein were present in all layers of the human colon. The dense expression of Panx1 on the submucosal and myenteric ganglia suggests that Panx1 may participate in neural control of colonic motility, secretion, and blood flow. Pannexin-1 has been demonstrated in hippocampal CA3 pyramidal neurons and retinal ganglia where it regulates neuronal ATP release, and promotes autocrine signaling via purinergic receptors. Extensive studies in guinea pig small intestine have shown that in the enteric nervous system, ATP released from enteric neurons mediates fast synaptic potentials via ionotropic ligand-gated P2X receptors and slow synaptic potentials via G-protein coupled P2Y receptors.

Taken together, this suggests that Panx1-mediated ATP release may exist along side of synaptically released ATP and represent a parallel paracrine signaling system between enteric neurons.

In the nematode *C. elegans*, Panx-like innexins in the intestinal smooth muscle cells form gap junctions and propagate intercellular calcium waves, which control the intestinal motor steps and regulate pacemaker cell activity. As Panxs do not form gap junctions in vivo, it remains unclear whether Panx1 found on colonic smooth muscle has similar action as its invertebrate counterpart. However, as intercellular calcium waves can propagate directly through gap junction channels, as well as indirectly by release of ATP onto extracellular purinergic receptors, Panx1 on the colonic smooth muscle cells may have a role in calcium permeability and synchronization between colonic smooth muscle cells.

In this study, Panx1-ir was found on the columnar epithelial cells and goblet cells of the colon. Mucosal ATP plays an important mechanosensory role in the initiation and propagation of enteric reflexes. In mouse taste buds, Panx1 hemichannels on the taste
receptor cells release ATP in response to gustatory stimuli, and ATP further stimulates other taste cells to release serotonin.\textsuperscript{27,28} We can speculate that Panx1 in the colonic epithelial cells may be involved in ATP-mediated sensory transduction. Additional pressure of food passing through the lumen may contribute to this mechanism as Panx1 is activated upon mechanical stress.\textsuperscript{6,26} In addition, previous studies have found Panx1 channels on the tracheal epithelial cells and goblet cells, where Panx1-mediated ATP release is thought to regulate mucociliary clearance.\textsuperscript{10} Ransford \textit{et al.}\textsuperscript{10} show that Panx1-ir is concentrated around the edges of cells in the airway in a similar manner to the immunohistochemistry data of this study. Adenosine triphosphate has also been shown to stimulate mucin secretion in respiratory epithelial cells\textsuperscript{29,30} and in an intestinal goblet cell line.\textsuperscript{31} This suggests a further role for Panx1 on intestinal goblet cells as a regulator of mucin secretion.

In this study, the most prominent Panx1-ir was observed on the endothelium of blood vessels and at the edges of erythrocytes. This is consistent with previous studies suggesting that Panx1 on erythrocyte

| Table 2 Comparison of Panx1 immunoreactive density in control, UC, and CD |
|------------------|-----|-----|-----|
| Cell type       | Control | UC  | CD  |
| Mucosa          |       |     |     |
| Glandular epithelium | +         | Damaged | + or damaged |
| Non-lymphoid leukocytes | +         | +++   | – +++   |
| in lamina propria |     |     |     |
| Mucosal blood vessels | +         | +++   | – +++   |
| Muscularis mucosae | +         | +     | +     |
| Submucosa       |       |     |     |
| Submucosal ganglia |     |     |     |
| Nerve cell bodies | ++      | +     | +     |
| Nerve fibers (varicosities) | +++ | +++ | +++ |
| Submucosal blood vessels | +++ | +++ | +++ |
| Endothelium    | +++   | +++ | +++ |
| Smooth muscle  | 0 – + | + | + |
| Red blood cells | +++   | +++ | +++ |
| Non-lymphoid leukocytes | + | +++ | + |
| Muscularis externa |       |     |     |
| Myenteric ganglia |       |     |     |
| Nerve cell bodies | ++      | 0 – + | – + – – |
| Nerve fibers (varicosities) | +++  | 0 – + | – + +++ |
| Circular muscle | +     | + | + |
| Longitudinal muscle | + – ++ | – – +++ | – – |

0, no immunoreactivity; ++++, strong, ++ moderate and + weak immunoreactivity.

Figure 5 Immunoreactivity of Panx1 protein (Panx1-ir) of control (A and B), UC (C and D), and CD (E and F) colonic smooth muscle. Myenteric ganglia (mg) between circular muscle (CM) and longitudinal muscle (LM) had strong brown Panx1-ir (A and B). There was little Panx1-ir exhibited in the mg (dotted outline) of UC (C) compared with control. Strong Panx1-ir can still be observed on blood vessels (bv) within the muscle layer of UC (C and D). Strong Panx1-ir can be seen on the mg, which seems to show less tissue (E). Serosa (S) of CD is larger in size compared with control serosa (F). Scale bars represent 50 μm.

© 2013 John Wiley & Sons Ltd
membranes responds to low oxygen tension, and on endothelial cells may mediate ATP release in response to thrombin. Release of ATP from these cells is potentially important in regulating vessel tone and peripheral resistance. Thus, it can be speculated that Panx1 plays an important role in the regulation of blood flow and oxygen supply to intestinal tissue.

Inflammatory bowel disease is a chronic and debilitating intestinal disorder, which is likely related to an immune system deficit as an underlying cause. Ulcerative colitis is often associated with episodic inflammation localized to the mucosa of the colon, whereas CD is not limited to the colonic mucosa and can affect other parts of the digestive tract. We hypothesize that Panx1 is involved in the pathogenesis of IBD as we have shown that significantly increased numbers of Panx1-ir inflammatory cell infiltration occur in UC and CD specimens. Previous studies have demonstrated that Panx1 and P2X7 receptors in mouse macrophages form a signaling complex, and activation of P2X7 by ATP triggers rapid opening of Panx1. Pannexin-1 involvement in inflammation is further supported by the evidence that probenacid, a non-selective Panx channel blocker used in the treatment of gout inflammation, inhibited ATP release from Xenopus oocytes expressing Panx1 channels.

Although the studies by Woehrle et al. have demonstrated that Panx1 mediates ATP release and facilitates intercellular communication between T cells and antigen-presenting cells, particularly in conditions of hypertonic stress, Panx1-ir was either absent or below detectable levels in lymphocytes in our study. This discrepancy may result from a number of issues. Firstly, the studies by Woehrle et al. are carried out in Jurkat cells and CD4+ T cell under culture conditions. Pannexin-1 forms ATP release channels only in activated T cells due to elevated cytosolic calcium levels, but not in inactivated T cells under culture conditions. As activated lymphocytes are usually present at low levels in peripheral blood and local tissues, Panx1-ir was not detected in paraffin-embedded tissues. Secondly, Panx1 was diffusely expressed in the cytoplasm of several cell types in the human colon. Therefore, it is possible that diffuse cytoplasmic Panx1 staining was masked by the dense staining of the large nucleus in lymphocytes, as only a faint ‘ring’ of cytoplasm can be seen on these cells.

In this study, we found a marked down-regulation of Panx1 in myenteric ganglia of UC, which was supported by a significant reduction in the levels of mRNA for Panx1 in UC muscularis samples. The density of Panx1-ir was also reduced in UC submucosal ganglia, but to a lesser extent compared with myenteric neurons. The reduction or loss of Panx1-ir occurred in all five specimens from different UC patients in this study, which contrasted with the study by Gulbransen et al. showing no changes in Panx1-ir in the myenteric plexus from UC subjects. However, our findings were highly consistent with a recent publication where Bernardini et al. have demonstrated that the densities of HuC/D+ neurons and S100β+ glial cells are reduced by 61% and 38%, respectively, in the myenteric ganglia of UC patients, thus we attribute the reduced Panx1 expression to enteric neuronal loss in UC myenteric ganglia or words to that effect would be appropriate.

Although inflammatory lesions in UC are limited to the mucosal region, colonic motility dysfunction is a common symptom in UC patients. Previous functional studies by us and other researchers have shown that the contractile response of human colonic circular muscle to tachykinins is reduced in UC. The reduction in Panx1 in myenteric neurons may be a significant factor for the reduced contractility of UC samples, as Panx1 is suggested to regulate calcium release from the endoplasmic reticulum stores in many cell types, including neurons. The present data supports previous studies showing that IL-1β is elevated in UC circular muscle where it induces production of hydrogen peroxide, resulting in a depletion of calcium from intracellular stores. There is increasing evidence that mast cells may participate in the pathogenesis of IBD. A significant increase in the number of mast cells has been found in all layers of the colon wall, including muscularis externa, in patients with UC. Mast cells reduce myenteric neuron survival in culture and may be involved in fibrogenesis and neuroplasticity in IBD. Taken together, the marked down-regulation of Panx1-ir in myenteric neurons in this study may be the consequence of inflammation-induced enteric neuronal and glial cell death, which is in accordance with the finding that activation of neuronal P2X7 receptor–Panx1 complex mediates death of enteric neurons during colitis.

### Table 3 Summary of Panx1 gene and protein expression in UC and CD in comparison with control

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>UC Muscularis</th>
<th>UC Mucosa</th>
<th>CD Muscularis</th>
<th>CD Mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>[by Real-time PCR]</td>
<td>↓** 0</td>
<td>↓* 1</td>
<td>↓* 1</td>
<td>↓* 1</td>
</tr>
<tr>
<td>Protein expression</td>
<td>0 0</td>
<td>↓*** 0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001, Mann-Witney test.
The number and the size of blood vessels were significantly increased in UC mucosal and submucosal regions, which were accompanied by an apparent increase in inflammatory cell infiltration in the mucosa, where a morphologic derangement occurred. The destruction of the mucosal tissue is thought to be a consequence of eosinophil infiltration and degranulation.48 However, the significant increase in the number and size of blood vessels in UC mucosa was not mirrored by the present quantitative data. That is, the level of Panx1 gene and protein expression in mucosa showed no difference between control and UC. It is possible that the apparent loss of Panx1-ir-positive epithelium and crypts may have effectively masked the increase in Panx1-expressing blood vessels and inflammatory cells in UC mucosa.

The decrease in Panx1 gene expression in CD muscularis was mirrored by a reduction in its protein expression. Unlike UC, the density of Panx1-ir in CD myenteric plexus remained unchanged compared with control. However, the ganglion cell bodies differed from control and appeared damaged (Fig. 5), implying that enteric neuronal cell death has occurred in CD patients, which was in line with the findings by Gulbransen et al.17, showing a marked loss of Panx1-ir in the myenteric plexus from three CD subjects. It has been reported that intestinal inflammation causes initial enteric neuron death, but subsequent axon outgrowth from surviving neurons restores innervation to the gut wall,49 which may explain the dense Panx1-ir observed in the myenteric plexus of CD patients in this study. Of note, the degree and region of inflammation varied considerably between the CD patients. An increased serosal thickness, which is thought to be the result of local chronic inflammation,50 was observed in three of six CD specimens, which is evidence for transmural inflammation, as a distinguishing feature of CD.

The down-regulation of Panx1 gene expression in CD mucosa was not reflected at the protein level. The disparity between gene and protein results was also seen in UC muscularis (Table 3). This difference is likely to be caused by the presence of high levels of erythrocytes in inflamed tissues. As gene transcription does not occur in erythrocytes due to the lack of nuclei, Panx1 from erythrocytes would not have contributed to the gene expression studies, but would have been accounted for in the protein expression studies. Thus, due to the nature of Panx1 expression patterns, it is important to use combined techniques, such as this study, to examine gene and protein expression, as well as cellular distribution.

In conclusion, this study has further characterized Panx1 in the colon, most notably in IBD. The high density and broad cellular localization of Panx1 in the colon indicates that Panx1 channels may be important in mediating a variety of gut functions. Complimentary techniques have shown decreases in Panx1 expression in both UC and CD, suggesting that Panx1 may be involved in the pathophysiology of IBD. These findings may provide new directions into the treatment of dysmotility and inflammation associated with IBD.

ACKNOWLEDGMENTS
We thank the Histology and Microscopy Unit for technical assistance and Emma Song and Jason Le for specimen collection.

FUNDING
This study was supported by a grant from the National Health and Medical Research Council of Australia [568861].

CONFLICT OF INTERESTS
The authors have no competing interests.

AUTHOR CONTRIBUTION
EFD performed the research, analyzed the data, and wrote the article; SLS & PPB designed the research study, and contributed essential reagents; IM technical support, and data collection; DSP, DZL & DWK provided human specimens and patient information; LL designed the research study, wrote the article; SLS & PPB designed the research study, and contributed essential reagents and tools.

REFERENCES
1 Baranova A, Ivanov D, Petras N et al. The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. Genomics 2004; 83: 706–16.

8 Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1β release by the ATP-gated P2X7 receptor. EMBO J 2006; 25: 5071–82.


26 Huang YJ, Maruyama Y, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD. The role of pannexin 1 hemichannels in ATP release and cell–cell communication in mouse taste buds. Proc Nat Acad Sci USA 2007; 104: 6436–41.


41 Menzies JRW, McKeel R, Corbett AD. Differential alterations in tachykinin NK2 receptors in isolated colonic


49 Gougeon PY, Lourenssen S, Han TY, Nair DG, Ropeleski MJ, Blennerhassett MG. The pro-inflammatory cytokines IL-1β and TNFα are neurotrophic for enteric neurons. *J Neurosci* 2013; *33*: 3339–51.