EPITHELIAL PROLIFERATION AND REPAIR

Activation of caspases in intestinal villus epithelial cells of normal and nematode infected rats

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Background: Small intestinal epithelial cells (IEC) show apoptosis in physiological turnover of cells and in certain inflammatory diseases.

Aims: To investigate the role of caspases in the progression of IEC apoptosis in vivo.

Methods: IEC were separated along the villus-crypt axis from the jejunum of normal and *Nippostrongylus brasiliensis* infected rats at 4°C. Caspases were examined by a fluorometric assay method, histochemistry, and immunoblotting.

Results: Villus cell rich IEC from normal rats exhibited a high level of caspase-3-like activity whereas activities of caspase-1, -8, and -9 were negligible. Immunoblotting analysis of villus cell rich IEC revealed partial cleavage of procaspase-3 into a 17 kDa molecule as well as cleavage of a caspase-3 substrate, poly(ADP-ribose) polymerase (PARP), whereas in crypt cell rich IEC, caspase-3 cleavage was less significant. Caspase-3 activity was also observed histochemically in villus epithelium on frozen sections of the normal small intestine. IEC prepared at 4°C did not reveal nuclear degradation whereas subsequent incubation in a suspension at 37°C induced intense nuclear degradation within one hour in accordance with increases in active caspase-3. This apoptosis was partially suppressed by the caspase inhibitor Z-VAD-fmk. Nematode infected animals showed villus atrophy together with significant increases in levels of caspase-3 in IEC but not of caspase-1, -8, or -9.

Conclusion: Caspase-3 may have an important role in the physiological replacement of IEC as well as in progression of IEC apoptosis induced by nematode infection.

In vitro studies have shown that apoptosis occurs in epithelial or endothelial cells when these cells are experimentally displaced from the extracellular matrix. This detachment induced apoptosis, or anoikis, was suppressed by replating the dissociated cells within a short time of suspension.¹⁻³ The mechanism responsible for this detachment induced apoptosis remains unclear but it has been ascribed to loss of integrin mediated survival signals derived from the extracellular matrix.¹⁻³ Detachment induced apoptosis has important roles in maintaining or modulating the normal epithelial architecture, such as those of the skin⁴ and the gastrointestinal tract,⁵ and in involution of the mammary gland.³ In the gastrointestinal tract, there are well defined zones of proliferation and migration of epithelial cells, and cells are finally lost by shedding from the distal end of the migration route with evidence of apoptosis.⁵ Normal colonic crypt epithelial cells undergo apoptosis rapidly in vitro due to the loss of anchorage during isolation at 37°C, while isolation at low temperatures can suppress this apoptosis.⁶

A number of studies have indicated that the final stage of apoptosis involves activation of caspases, which are constitutively expressed in most cells as single chain proenzymes.^{7,8} During apoptosis triggered by signals such as cytochrome c released from mitochondria or ligation of the death receptor Fas or tumour necrosis factor receptor-1, procaspases are activated to fully functional proteases by proteolytic cleavage. Cytochrome c induces caspase-9 activation.⁹ Caspase-8 is activated early during Fas mediated apoptosis.¹⁰ Caspase-1 has also been reported to be activated in Fas mediated apoptosis.¹¹ Caspase-3 acts downstream of other caspases and is responsible either partially or totally for proteolytic cleavage of many key proteins such as lamins, poly(ADP-ribose) polymerase (PARP), and beta-catenin.^{7,8} Grossmann and colleagues¹² reported that isolated normal colonic epithelial cells showed rapid activation of caspase-6 and -3 before DNA fragmentation. Caspase activation has also been reported in certain cell

types such as lens epithelial cells and human epidermal cells when they terminally differentiate and lose their nucleus and other organelles.^{13,14}

In the small intestine, cells generated from stem cells at the base of the crypt differentiate into absorptive cells and are finally lost from the tips of villi, resulting in replacement of lining cells every two to three days.⁵ Apoptotic cells are normally observed at the tips of villi as well as in crypts.⁵ In certain inflammatory conditions such as coeliac disease, nematode infections, and graft versus host disease, numbers of apoptotic nuclei were reported to be increased in villus epithelial cells,¹⁵⁻¹⁷ indicating that apoptosis has important roles not only in physiological replacement of villus epithelial cells but also in pathological conditions. In this respect, it is of interest to determine whether caspases are involved in the turnover of villus epithelial cells in the small intestine. In the present study, we examined activities of caspase-1, -3, -8, and -9 in epithelial cells of the small intestine of normal rats as well as in rats infected with the nematode *Nippostrongylus brasiliensis*, which induces partial villus atrophy together with enhancement of apoptosis in villus epithelial cells.¹⁶

MATERIALS AND METHODS

Animals

Specific pathogen free male Brown Norway/Sea (BN) rats were purchased from SLC Inc. (Shizuoka, Japan).

Abbreviations: IEC, intestinal epithelial cells; PARP, poly(ADP-ribose) polymerase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BrdU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; ALP, alkaline phosphatase; VCU, villus-crypt units.

Nematode infection

Animals, eight weeks of age, were injected subcutaneously with 2000 *N. brasiliensis* third stage larvae, as described elsewhere.¹⁶

Preparation of intestinal epithelial cells (IEC)

The process of separation of IEC was carried out at 4°C in EDTA-Hanks' solution (Ca²⁺, Mg²⁺ free Hanks' balanced salt solution supplemented with 10 mM HEPES, pH 7.3, and 0.5 or 1.0 mM EDTA). To obtain serial fractions of epithelial cells along the crypt-villus axis (fractions A–D), a segment of proximal jejunum was removed, opened longitudinally, and cut into segments 1 cm in length. After a brief wash in phosphate buffered saline, four pieces of tissue were placed into a 15 ml tube containing 4 ml of 0.5 mM EDTA-Hanks' solution. After 20 minutes, epithelial cells (fraction A) were separated by vigorous shaking of the tubes for five seconds and tissues were transferred into another tube containing 0.5 mM EDTA-Hanks' solution. This process was repeated two more times with a 20 minute interval to obtain fractions B and C. After separation of fraction C, tissues were transferred into a tube containing 1.0 mM EDTA-Hanks' solution, and after 30 minutes the remaining epithelial cells (fraction D) were separated by vigorous shaking of the tube for 20 seconds. Detached epithelial cells in tubes A–D were collected by centrifugation at 600 *g* for five minutes at 4°C, and cell pellets were kept at –80°C until use. Histological examination of the tissue after collection of fraction D showed that villus epithelial cells were separated completely whereas epithelial lining cells in the lower part of crypts were still attached to the tissue in approximately half of the crypts. The basal lamina of the epithelium was intact and lamina propria cells were retained in the tissue. To obtain total IEC, tissues were immersed in cold 1.0 mM EDTA-Hanks' solution for one hour, and epithelial cells were separated by vigorous shaking of the tube for 20 seconds.

Ex vivo incubation of IEC

IEC were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with or without 5% fetal calf serum (FCS), and incubated (3×10⁶/ml) in petri dishes at 37°C under a 5% CO₂ atmosphere. In some experiments, the serine protease inhibitor TLCK (Sigma, St Louis, Missouri, USA), the caspase inhibitor Z-VAD-fmk (Peptide Institute Inc., Osaka, Japan), or an equivalent amount of DMSO was added to the culture medium.

Tissue and cell extract

To minimise the possibility of activation of caspases during the extraction process, tissue extracts were prepared as quickly as possible at 4°C. Briefly, tissues were resected from the glandular stomach, jejunum (20 cm from the pyloric ring), ileum (10 cm oral from the ileum end), and transverse colon, washed briefly in cold phosphate buffered saline, and cut into small segments. Approximately 100 mg of tissue were immersed in 1 ml of extraction buffer (25 mM HEPES, pH 7.3, containing 0.5% NP40, 1 mM DTT, 2 mM EDTA, 2 mM PMSF, 20 mg/ml leupeptin, 5 mg/ml pepstatin, and 5 mg/ml aprotinin) and immediately vortexed for 60 seconds. After discarding the tissue, the crude extracts were further subjected to one cycle of freezing-thawing and centrifuged at 15 000 *g* for five minutes at 4°C. The supernatant was stored at –80°C until use. Cytosolic extracts of IEC were prepared by two cycles of freezing-thawing of the cells in 1 ml of cold extraction buffer. Protein concentration was determined by the Bradford method.

Fluorometric assay of caspases

Activities of caspases were measured by a fluorometric method, as described previously,^{18–20} with minor modifications.

The fluorogenic synthetic substrates Ac-DEVD-AMC, Ac-YVAD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC, and specific inhibitors Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-IETD-CHO, and Ac-LEHD-CHO were purchased from Peptide Institute Inc. Briefly, appropriately diluted cell or tissue extracts were added to caspase reaction buffer (100 mM HEPES, pH 7.5, 10 mM DTT, 10% sucrose, 0.1% CHAPS) containing 10 mM substrate, and the reaction mixtures were monitored continuously at 26°C in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Standard curves were generated with AMC.

Histochemical demonstration of caspase-3 activity

A segment of jejunum was removed and snap frozen in liquid nitrogen. Cryostat sections (10 µm) were cut and mounted on glass slides. Immediately after fixation in acetone for 10 seconds and air drying, sections were overlaid with 7 µl of a solution containing 0.5% (w/v) carboxymethylcellulose sodium salt, high viscosity (Sigma Chemical Co.), 20 mM Ac-DEVD-AMC, 0.01 M phosphate buffer, pH 7.4, 10 mM DTT, and 0.1% CHAPS, with or without Ac-DEVD-CHO, and covered with a glass coverslip (18×18 mm). Sections were immediately observed at room temperature under a fluorescence microscope (Olympus excitation filter UG-1 peaked at 370 nm and absorption filter L-420). Carboxymethylcellulose yielded high viscosity to reduce dispersion of fluorescent AMC on tissue sections during observation.

Western blotting

Cell pellets or tissues cut into small pieces were directly immersed in sodium dodecyl sulphate sample buffer containing 5% 2-ME, and heated at 95°C for five minutes. Proteins were separated on 4–20% gradient sodium dodecyl sulphate-polyacrylamide gels, and electrotransferred onto Immobilon P membranes (Millipore Corp., Bedford, Massachusetts, USA). After blocking with 5% non-fat dry milk, immunodetection was carried out using antibodies against caspase-3, PARP, actin, and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), followed by incubation with Envision (Dako, Carpinteria, California, USA).

Alkaline phosphatase (ALP) activity

ALP activity was determined according to the method described previously,²¹ with *p*-nitrophenylphosphate as substrate.

Bromodeoxyuridine (BrdU) incorporation assay

Rats received an intraperitoneal injection of BrdU (100 mg/kg), one hour before sacrifice, and IEC were isolated as described above. IEC were resuspended in a solution containing 10 mM Tris HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 1 mM DTT, homogenised gently in a glass homogeniser, and nuclei were collected by centrifugation. Isolated nuclei were seeded onto 96 well plates (5×10³/well) and dried at 60°C for two hours. The amount of BrdU was measured with an ELISA kit (Amersham Life Science Ltd., Amersham Place, UK) according to the manufacturer's instructions.

Nuclear staining

Hoechst 33258 (10 µg/ml; Sigma) dissolved in DMEM was added to IEC cultures 20 minutes before harvesting of cells. Nuclei were observed under a fluorescence microscope.

DNA extraction and electrophoresis

Total DNA was extracted by the phenol/chloroform method and electrophoresed on a 2% agarose gel. The gel was stained with ethidium bromide and DNA bands were visualised by ultraviolet fluorescence.

Figure 1 Caspase-3-like activities in the normal rat intestinal tract. (A) DEVD-AMC cleavage activities (mean (SD) of four animals) in extracts of the stomach, jejunum, ileum, and colon. (B) Effects of inhibitors on DEVD-AMC cleavage activities in jejunum extracts. The inhibitors indicated were added at 100 nM to 1.5 μ g/ml extracts of the jejunum, and DEVD-AMC cleavage activity was determined after 20 minutes. (C) Western blotting analysis of extracts of the stomach (S), jejunum (J), ileum (I), and colon (C) for caspase-3: 32 kDa bands represent procaspase-3; 17 kDa bands represent cleaved caspase-3, indicative of active caspase-3; and 19 kDa and other bands are intermediate products in the processing of caspase-3.

Partial purification of caspase-3 from IEC

Total IEC were obtained as described above from the whole length of the small intestine. IEC extracts were applied to a Mono Q chromatography column (Pharmacia, Uppsala, Sweden) and eluted with increasing NaCl concentrations up to 0.5 M. The fraction with peak DEVD-AMC cleavage activity was further purified by Superose 12 (Pharmacia) gel filtration. The final enzyme preparation had a specific activity of 57 000 pmol/min/mg, and western blotting analysis showed a 32 kDa proenzyme and 19 and 17 kDa cleaved caspase-3 but sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed contamination by many other protein bands.

In vitro PARP cleavage

PARP isolated from bovine thymus (Biomol Research Laboratories Inc., Plymouth Meeting, Pennsylvania, USA) (17 ng/ μ l) was incubated with partially purified IEC caspase-3 (0.5 U/ μ l) in caspase reaction buffer with or without 10 μ M DEVD-CHO at 37°C. Caspase-3 1 U was defined as the amount capable of degradation of 1 pmol DEVD-AMC/min.

Histology

Animals were sacrificed by excess inhalation of ether. A segment of the jejunum 15–20 cm distal to the pyloric ring was removed, opened longitudinally, fixed in 4% buffered formalin overnight, and embedded in paraffin in such a position that histological sections could be cut perpendicular to the luminal surface. Measurements were carried out on haematoxylin and eosin stained sections cut at 5 μ m. Twenty villus-crypt units (VCU), which were cut exactly perpendicularly, were selected from three sections per animal, and lengths of villi and crypts were measured directly under a microscope using an ocular lens with a micrometer. The average length in 20 VCU was employed as the representative length in a given animal, and the means (SD) of five animals were calculated. The surface to volume ratio was measured as described by Dunnill and Whitehead.²² Briefly, sections were projected onto a white board on which was drawn 15 lines of equal length as

Figure 2 Caspase-1, -8, and -9 activities examined by cleavage of fluorogenic peptides YVAD-AMC, IETD-AMC, and LEHD-AMC, respectively, in extracts of the stomach (S), jejunum (J), and colon (C). Protein extracts (10 μ g/ml) were added to 10 μ M fluorogenic substrate in the presence or absence of the indicated inhibitors (1 μ M each). Data represent averages of duplicate assays.

Figure 3 Caspase-3-like activities in jejunal intestinal epithelial cells (IEC). (A) DEVD-AMC cleavage activities, alkaline phosphatase (ALP) activities (mean (SD) of four animals), and bromodeoxyuridine (BrdU) incorporation level (one representative plot of two independent experiments) in serial fractions of IEC (A–D) and residual tissue. (B) Western blotting analysis for proliferating cell nuclear antigen (PCNA) and caspase-3 in IEC fractions (A–D) and residual tissue (R). Immunoblotting analysis for actin was performed to control for equal loading of protein. (C) Effects of inhibitors on DEVD-AMC cleavage activity of lysates of total IEC. Lysates (1.25 μ g/ml) were incubated with 10 μ M DEVD-AMC in the presence or absence of DEVD-CHO at the indicated concentrations.

described by Weibel.²³ Magnification was such that the length of each line corresponded to a length (L) of 1.4×10^{-2} cm. The section was projected at random onto the template lines and the number of times the lines cut the mucosal surface (c) and the number of times the end points of the lines fell on mucosal tissue (h) were counted from 20 randomly selected fields/animal. The ratio c/Lh gives an index of the volume to surface ratio, and the mean number of h per field gives an index of mucosal volume.

Statistics

Student's *t* test was employed for statistical analysis, and a *p* value of less than 0.5 was considered significant.

Figure 4 Histochemical demonstration of DEVD-AMC cleavage activity in the jejunum. (A) The villus epithelium showed blue AMC fluorescence ($\times 50$). (B) AMC fluorescence was observed mainly in the cytoplasm of villus epithelial cells with no fluorescence in the nucleus or goblet cell mucins. Slightly greenish autofluorescence was observed in lamina propria cells ($\times 100$). (C) AMC fluorescence did not appear in the presence of $10 \mu\text{M}$ DEVD-CHO ($\times 50$).

RESULTS

Caspase activities in the normal gastrointestinal tract

Caspase activities were examined in tissue extracts from the glandular stomach, jejunum, ileum, and colon of normal rats using fluorogenic substrates DEVD-AMC, IETD-AMC, LEHD-AMC, and YVAD-AMC for caspase-3, -8, -9, and -1, respectively. To avoid activation of caspases during extraction, the procedures were carried out rapidly at 4°C (see methods). Unexpectedly, significantly high levels of caspase-3-like activity were detected in extracts from the normal jejunum and ileum, although activity was negligible in the stomach and colon (fig 1A). Caspase-3-like activity in the jejunum was suppressed significantly in the presence of the specific inhibitor DEVD-CHO (fig 1B). Immunoblotting of extracts from the jejunum and ileum showed 19 and 17 kDa cleaved forms as well as a 32 kDa proenzyme of caspase-3, indicating that caspase-3 was partially activated (fig 1C). Extracts from the stomach and colon showed only caspase-3 proenzyme.

Cleavage activities for IETD-AMC and LEHD-AMC were also detected at low levels in the jejunum extracts but their

activities were markedly suppressed not only by the respective specific inhibitors but also by DEVD-CHO, suggesting that these activities may have been derived from caspase-3, and not from caspase-8 or -9 (fig 2). YVAD-AMC cleavage activity was not detected.

To determine whether caspase-3 activity in the small intestine is derived from IEC, serial fractions of epithelial cells (A–D) were prepared at 4°C from the jejunum (see methods). ALP activity, a marker for differentiated absorptive cells, was highest in extracts of fraction A and declined gradually in the subsequent fractions (fig 3A). In contrast, BrdU incorporation was high in fraction D, suggesting that this fraction was rich in crypt lining cells, and fractions A–C were rich in villus epithelial cells (fig 3A). High levels of caspase-3-like activity were observed in fractions A–C whereas those of crypt cell rich fraction D and the residual tissue were significantly lower than those of fractions A–C (fig 3A). Western blotting analysis of epithelial cell fractions also showed that levels of expression of the 17 kDa cleaved form of caspase-3 were high in fractions A–C and low in fraction D. On the other hand, PCNA expression was higher in fraction D than in fractions A–C (fig 3B). Caspase-3 activity in separated total IEC was inhibited with as little as 1 nM DEVD-CHO (fig 3C), and the K_m for DEVD-AMC estimated from Lineweaver-Burk (double reciprocal) plots was 17.7 mM .

To confirm that the majority of caspase-3-like activity in the normal small intestine was derived from villus epithelial cells, caspase-3-like activity was analysed histochemically on cryostat sections of snap frozen normal jejunum. AMC fluorescence appeared over villus epithelium immediately after incubation of cryostat sections with substrate at room temperature (25°C) (fig 4A). This activity was localised mainly in the cytoplasm apical to the nucleus, while nuclei and goblet cell vacuoles were negative (fig 4B). The activity was completely inhibited in the presence of $10 \mu\text{M}$ DEVD-CHO (fig 4C). Some lamina propria cells exhibited slightly greenish autofluorescence (fig 4B, C), and this could be distinguished from the blue fluorescence derived from AMC.

Caspase-3 was partially purified from extracts of IEC (see methods), and its PARP cleavage activity was examined in vitro. Addition of IEC caspase-3 preparation to PARP induced rapid cleavage of the latter to an 85 kDa degraded form (fig 5A). Immunoblotting analysis of PARP in IEC extract showed

Figure 5 (A) Cleavage of poly(ADP-ribose) polymerase (PARP) by partially purified caspase-3 from intestinal epithelial cells (IEC). Purified bovine PARP was incubated with IEC caspase-3 for the indicated times in the absence or presence of $10 \mu\text{M}$ DEVD-CHO, and PARP cleavage was analysed by western blotting (C=no addition of caspase-3 to PARP). (B) Western blotting analysis for PARP in IEC extracts (fractions A–D). PARP, bovine PARP; PARP+casp-3, bovine PARP incubated with IEC caspase-3 for 60 minutes.

Figure 6 Induction of intestinal epithelial cell (IEC) apoptosis ex vivo. (A) IEC separated from the jejunum were suspended in DMEM (2×10^6 cells/ml) with or without 5% fetal calf serum (FCS) and incubated at 4°C or 37°C. Cell nuclei were stained with Hoechst 33258, and nuclear degradation was examined under a fluorescence microscope. Data are mean (SD) of quadruplicate cultures. (B) DEVD-AMC cleavage activities in the cytosolic extracts and culture supernatant (Sup) of IEC incubated at 37°C without serum. (C) Liberation of caspase-3 from apoptotic cells into culture supernatant. Culture supernatants (B) were analysed by western blotting for caspase-3.

Figure 7 Effects of the broad range caspase inhibitor VAD-fmk or serine protease inhibitor TLCK on induction of apoptosis in ex vivo culture of intestinal epithelial cells (IEC). IEC (2×10^6 /ml) were preincubated with VAD-fmk or TLCK at the indicated concentrations for 30 minutes at 4°C, and incubated at 37°C for one hour with the same concentrations of inhibitors. Cultures without inhibitors contained 0.5% DMSO, which was equivalent to the amount in those containing 100 μ M VAD-fmk. (A) Nuclear degradation was determined by cell staining with Hoechst 33258. Data are mean (SD) of quadruplicate cultures. (B) Western blotting analysis for caspase-3 in cells and supernatant (Sup) of IEC cultures.

the presence of the intact as well as the degraded form of PARP (fig 5B).

Induction of IEC apoptosis in vitro

IEC separated at 4°C revealed no nuclear degradation. When these cells were transferred to culture dishes and incubated in

Figure 8 Caspase-3-like activities in the jejunum after *N. brasiliensis* infection. Extracts were obtained from (A) the total tissue, (B) upper villus epithelial cells (fraction A), or (C) remaining epithelial cells obtained after separation of fraction A, and DEVD-AMC cleavage activities were determined. Data are mean (SEM) of five animals. Significantly different from day 0 (uninfected control): * $p < 0.05$, ** $p < 0.01$.

a suspension at 37°C, apoptotic nuclear degradation developed within one hour (fig 6A). Levels of caspase-3-like activity in cytosolic extracts were also increased after 20 and 40 minutes of incubation, and at 60 minutes and thereafter activity as well as the 17 kDa cleaved form of caspase-3 emerged and increased in the culture supernatant (fig 6B, C). On the other hand, there were no increases in activities of caspase-1, -8, or -9 after incubation at 37°C (data not shown). Addition of the caspase inhibitor VAD-fmk to the culture medium showed dose dependent partial suppression of nuclear degradation, while the serine protease inhibitor TLCK did not (fig 7A). VAD-fmk also suppressed the release of 17 kDa caspase-3 into the supernatant (fig 7B). DNA electrophoresis showed only slight suppression of the formation of oligonucleosomal fragments in the presence of VAD-fmk (data not shown). Addition of the specific caspase-3 inhibitor DEVD-fmk did not suppress apoptosis, at least up to a dose of 100 μ M. However, immunoblotting analysis indicated that caspase-3 activation proceeded even in the presence of DEVD-fmk, suggesting that DEVD-fmk was not efficiently absorbed in the cells (data not shown).

Activation of caspases in IEC after nematode infection

Previously, we reported that infection by the intestinal nematode *N. brasiliensis* induced increases in the number of TUNEL positive cells in the epithelium of the upper part of the villi together with reduction of villus length.¹⁶ In the present study, we examined whether caspase-3 is involved in the progression of apoptosis induced by nematode infection. Ten days post-infection when high worm burden was observed in a segment of the jejunum, significant reductions in villus length as well as surface to volume ratio were observed, and these alterations persisted at least until 20 days postinfection (table 1). In contrast, crypt length showed elongation. Levels of caspase-3 activity in total tissue extracts were increased significantly 10 days postinfection, while they returned to normal levels 20 days postinfection when the majority of worms had been rejected from the jejunum. IEC fraction A also revealed significant increases in caspase-3 activity, while IEC obtained

Table 1 Villus atrophy and crypt hyperplasia of the jejunum induced by *Nippostrongylus brasiliensis* infection

Time after infection (days)	No of worms ¹	Villus length ² (μ m)	Crypt length ² (μ m)	Surface:volume ratio ³ (c/Lh)	Estimated volume ³ (h)
0	0 (0)	557.1 (21.4)	200.4 (9.2)	68.4 (4.6)	19.0 (0.8)
10	43.0 (29.7)	455.3 (38.4)**	273.6 (17.8)**	39.0 (5.3)**	19.1 (0.7)
20	3.0 (3.4)	488.6 (31.7)**	244.0 (12.8)**	53.2 (19.2)**	19.2 (0.9)

¹Numbers of worms emerging from a jejunal segment 25–29 cm from the pyloric ring in saline at 37°C for one hour were counted.

²Villus and crypt lengths were determined in paraffin embedded tissue sections obtained 15–20 cm from the pyloric ring.

³Surface:volume ratio and estimate of volume were determined as described in materials and methods.

All data are mean (SD) of five rats.

Significantly different from day 0 (uninfected control):** $p < 0.01$.

after separation of fraction A showed no significant increases (fig 8). Activities of caspase-1, -8, and -9 did not change significantly after infection (data not shown). DEVD-AMC cleavage activity in the small intestine was also examined histochemically on cryostat sections of normal rats and of those 7, 10, and 20 days postinfection. Despite the significant increases in caspase-3 activity in the IEC fraction A after infection detected by fluorometric assay, the histochemical AMC fluorescence pattern over villus epithelium in animals after infection was similar to that of controls, probably due to the low sensitivity of the DEVD-AMC histochemical method (data not shown).

DISCUSSION

The present study showed high levels of caspase-3-like activity in extracts of the normal small intestine, and this activity was derived from partially activated caspase-3 in villus epithelial cell rich IEC. In contrast, in crypt cell rich IEC, levels of activity and the 17 kDa cleaved form of caspase-3 were significantly lower than those in villus cell rich IEC. It is unlikely that procaspase-3 in the villus epithelium was activated during the extraction process as rapid extraction was carried out at 4°C immediately after tissue resection. Furthermore, caspase-3-like activity was demonstrated histochemically over villus epithelium in cryostat sections of snap frozen small intestine. These results indicated that partial activation of caspase-3 occurs after crypt lining cells differentiate into villus absorptive cells. The estimated K_m of IEC extracts for DEVD-AMC cleavage (17.7 mM) compared relatively favourably with that reported for caspase-3 purified from apoptotic cell extracts (K_m of 9.7–13.6 mM).¹⁸ Partially purified caspase-3 from normal IEC efficiently cleaved PARP, one of the target substrates of caspase-3, in vitro and IEC extracts revealed the cleaved form of PARP. These results indicated that at least some of the normal villus absorptive cells are already committed to apoptosis even before detachment from the basal lamina.

Despite the presence of active caspase-3 in normal IEC, the majority of cells revealed no morphological features of apoptosis. It seems that the presence of active caspase-3 in the cytoplasm may not necessarily be an indication of impending cell death as long as the cells are adherent to the villus basal lamina. It is possible that active caspase-3 in normal IEC is below a critical level that permits access of caspase-3 to the nuclear substrate. In fact, histochemical analysis demonstrated the presence of caspase-3-like activity in the cytoplasm, but not in the nucleus of IEC. However, isolated normal IEC were highly sensitive to apoptosis when cultured in suspension at 37°C. Nuclear degradation occurred as early as 20 minutes after incubation, concomitantly with significant increases in levels of caspase-3 activity. The parallel increase in caspase-3 activity with progression of nuclear degradation in suspension culture of IEC was consistent with the findings reported in human isolated colonic crypt cells which revealed DNA fragmentation 90 minutes after detachment, preceded by activation of caspase-6 and caspase-3.¹² The caspase inhibitor VAD-fmk however suppressed the detachment induced apoptosis of IEC only partially. This suggested that a catastrophic molecular event for execution of apoptosis had already begun when the concentration of the inhibitor reached an effective level in the cells. Alternatively, a death programme that may not depend on caspases may also be present in IEC, as suggested in sperm and chicken erythrocytes.²⁴ Nevertheless, these results showed that nuclear degradation takes places very quickly after IEC are detached from the basal lamina, and caspase activation is involved, at least partially, in this detachment induced apoptosis.

It has been reported that caspase-3 activation is induced by proteolytic cleavage of procaspase-3 through the action of upstream caspases such as caspase-1, -8, -9, and -10,^{25–27} or

granzyme B.^{28–29} However, the activities of at least caspase-1, -8, and -9 were negligible in IEC of normal as well as nematode infected animals. It was recently reported that RGD containing peptides, inhibitors of integrin-ligand interactions, induce direct induction of autoprocessing and enzymatic activity of procaspase-3.³⁰ Sträter and colleagues³¹ reported that detachment induced apoptosis of isolated human colonic crypt cells was partially inhibited when cells were in contact with collagen I coated membranes, and anti- $\beta 1$ integrin antibody caused a much higher rate of apoptosis. In our preliminary experiments however apoptosis of IEC was not suppressed in collagen I coated dishes. On the other hand, it has been reported that in the normal and neoplastic colon, mucosal expression of proapoptotic protein Bak was colocalised with sites of epithelial cell apoptosis.³² Thus members of the Bcl-2 family may have important roles in activation of caspase-3 in IEC. The mechanisms that trigger activation of caspase-3 before as well as after detachment of IEC from the basal lamina should be clarified in future studies.

Caspases have important roles not only in physiological cell death in the development or physiological turnover of cells in adult life but also in the pathogenesis of certain inflammatory diseases. Caspase-1 is crucially involved in immune mediated inflammation because of its pivotal role in regulation of cellular export of interleukin-1 β and interleukin-18.^{27–33–34} In Alzheimer's disease, caspase-3 was suggested to play an important role in both generation of neurotoxic amyloid β peptide as well as in the ultimate death of neurones by apoptosis.³⁵ Certain inflammatory disorders of the small intestine such as coeliac disease and parasite infections are associated with enhanced apoptosis in the IEC with crypt hyperplasia, the latter reflecting enhanced replacement of damaged IEC.^{15–16} The present results showed that nematode infection induced significant increases in caspase-3 activity in IEC. We previously reported that *N. brasiliensis* infection induced increases in apoptosis in BN rat IEC.¹⁶ Thus increases in caspase-3 activity may reflect accelerated apoptosis in IEC after nematode infection. However, it remains to be elucidated whether enhanced apoptosis in IEC is involved in the progression of villus atrophy.

Taken together, our results suggest that in the small intestinal epithelial cells partial activation of caspase-3 takes place after crypt cells are differentiated into villus absorptive cells. Caspase-3 may have important roles in physiological replacement of IEC as well as in the progression of IEC apoptosis induced by nematode infection.

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