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INVOLVEMENT OF DNA FRAGMENTATION OF ENTEROCYTES IN MUCOSAL INJURY TO A MOUSE JEJUNUM INCUBATED IN USSING CHAMBERS

EIKO INAGAKI-TACHIBANA[1], TAKAMITSU TSUKAHARA[2], KAZUHIKO KAJI[3], RYOJI EGUCHI[3], HIROAKI KANAZAWA[4], HISAYOSHI HAYASHI[5] and YUICHI SUZUKI[5]

[1] *Department of Nutritional Sciences, School of Nutritional Sciences, Nagoya University of Arts and Sciences, 57 Takenoyama, Iwasaki-cho, Nisshin 470-0196, Japan*
[2] *Kyoto Institute of Nutrition & Pathology, Kyoto 610-0231, Japan*
[3] *Laboratory of Cell and Molecular Biology of Aging and COE Program in the 21st Century, Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada 52-1, Surugaku, Shizuoka 422-8526, Japan*
[4] *Laboratory of Anatomy, School of Nursing, University of Shizuoka, Yada 52-1, Surugaku, Shizuoka 422-8526, Japan*
[5] *Laboratory of Physiology, School of Food and Nutritional Sciences, University of Shizuoka, Yada 52-1, Surugaku, Shizuoka 422-8526, Japan*

ABSTRACT

A mouse jejunum, when incubated *in vitro* in Ussing chambers, was found to exhibit morphological deterioration of the villi with denudation of the epithelia (*J Nutr Sci Vitaminol*, 51: 406, 2005). Our study examined the involvement of apoptosis in an intestinal injury model by a DNA ladder assay and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. Electrophoresis of mucosal DNA revealed ladders, indicating the occurrence of DNA fragmentation. Cells with TUNEL-positive nuclei were detected among the villus epithelial cells (enterocytes), whereas they are rarely seen among crypt epithelial cells. These features were evident within 1 h after the start of incubation. Apoptotic death of the enterocytes was thus involved in the destruction of villi when incubated in Ussing chambers.

Key Words: Apoptosis, DNA ladder, TUNEL staining, Villus, Intestinal barrier

INTRODUCTION

Intestinal epithelial cells have a very rapid turnover, with villus epithelial cells (enterocytes) at the tip of villi removed by apoptosis, a physiological process for eliminating unwanted and damaged cells.[1-2] Apoptosis of intestinal epithelial cells is important not only in physiological cell renewal, but is also implicated in several pathological conditions.[2-3] Enterocyte apoptosis has been demonstrated in ischemia/reperfusion injury,[4-7] and in the mucosal damage after traumatic brain injury.[8] In addition, an *ex vivo* culture of intestinal tissue has been shown to undergo apoptosis.[9]

Corrersponding author: Eiko Inagaki-Tachibana
Department of Nutritional Sciences, School of Nutritional Sciences, Nagoya University of Arts and Sciences, 57 Takenoyama, Iwasaki-cho, Nisshin, Aichi 470-0196, Japan
Phone: +81-561-75-2550, Fax: +81-561-73-8539, E-mail: e-ina@nuas.ac.jp

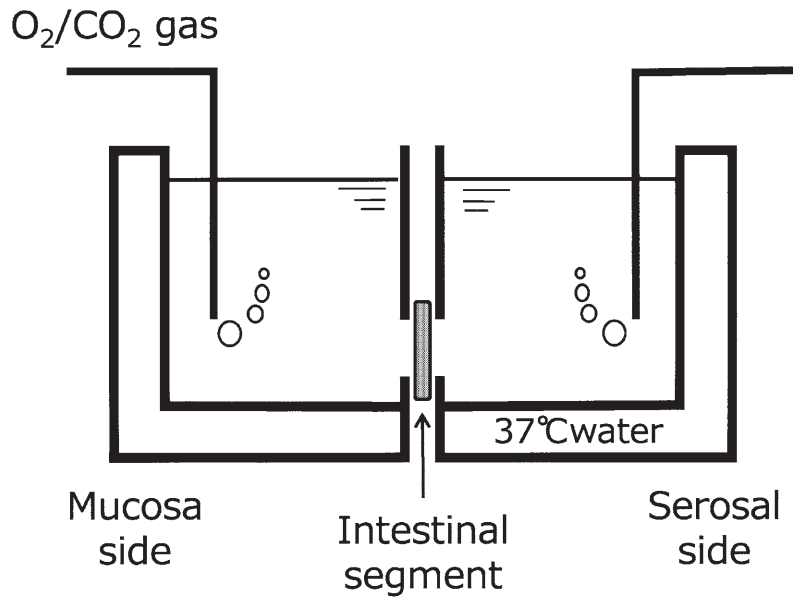

Fig. 1 Schematic drawing of Ussing chamber. Chamber had an exposed area of 0.2 cm^2 and contained 5 ml of bathing solution, which was bubbled with 95% O_2/5% CO_2 gas. The temperature was maintained at 37ºC in a water-jacketed reservoir.

We have previously demonstrated an injury to the small intestine when incubated in Ussing chambers.[10-11] The Ussing chamber (Fig. 1) has been used for *in vitro* studies of transport in the small intestine. Under this condition, the mucosa was disrupted, especially at the top of the villi, and that disruption was more severe in the proximal than in the distal small intestine, although the mucosal barrier function was not markedly reduced.[11] In the present study, we investigated the involvement of apoptosis in this intestinal injury model. We detected internucleosomal DNA degradation (one of the final events of apoptosis) by ladder formation with electrophoresis and by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL).

MATERIALS AND METHODS

Animals and solutions

All procedures used in this study were performed in accordance with the "Guiding Principles for the Care and Use of Animals" of the Nagoya University of Arts and Sciences. Male mice (30–40 g, 7–8 weeks old, Std:ddY; Japan SLC, Hamamatsu, Japan) were sacrificed by cervical dislocation between 10:00 and 14:00 h, and the small intestine from the ligament of Treiz was excised to about 3 cm below it. The segment was opened and mounted between Ussing chambers with an exposed area of 0.2 cm^2 (Fig. 1). The volume of the bathing solution on each side was 5 ml, and the solution temperature was maintained at 37ºC in a water-jacketed reservoir. The composition of the incubation solution (in mM) was NaCl (119), $NaHCO_3$ (21), KH_2PO_4 (0.6), K_2HPO_4 (2.4), $CaCl_2$ (1.2) and $MgCl_2$ (1.2), and was bubbled with 95% O_2/5% CO_2 (pH=7.4). The bathing solution on the mucosal side was supplemented with 8.5 mM mannose, and the serosal solution with 5 mM glucose, 2.5 mM L-glutamine and 1 mM β-hydroxybutyric acid as

metabolic substrates.

DNA ladder and TUNEL assays

At the end of various incubation times, the tissue was removed from the Ussing chamber and subjected to several treatments. The tissue was scraped with a scalpel to harvest a mucosal sample for the DNA ladder assay. The sample was homogenized with 100 µl of a lysis buffer consisting of 50 mM Tris-HCl, 10 mM EDTA and 0.5% SDS (pH=7.8). One hundred µg/ml of RNase was added, and the sample incubated for 3 h at 37ºC. Proteinase K (2 mg/ml, Takara Bio, Otsu, Japan) was then added, and incubation was continued overnight at 65ºC. DNA was purified with sequential phenol and phenol/chloroform/isoaminoalcohol extraction. DNA was precipitated with ethanol, resuspended in a TE buffer (10 mM Tris-HCl and 1 mM EDTA), and separated by electrophoresis on 2% agarose gel, before visualizing with ethidium bromide under ultraviolet light.

Histological studies were performed on the removed tissue that had been fixed in a neutral-buffered, 10% (v/v) formalin solution (pH 7.1), and then embedded in paraffin wax. Four-µm-thick sections were prepared for use in the hematoxylin-eosin staining or TUNEL assay. The latter was conducted with a commercially available *in situ* apoptosis detection kit (Takara Bio) according to the manufacturer's instructions.

Statistics

Group comparisons were analyzed by a one-way analysis of variance and a subsequent Dunnett's test. Data are each presented as the mean ± SE (n=number of animals).

RESULTS

DNA fragmentation was determined by a DNA laddering assay in the mouse jejunum incubated in the Ussing chamber (Fig. 2). In the normal jejunum (not shown) or in the tissue just after the start of incubation (0 min), DNA laddering was scarcely visible. However, multiples of 180-bp subunits detected in some, although not all, mucosal samples as early as 15 min, were always clear at 30 min and thereafter. To quantify the time-course characteristics of DNA fragmentation, the ratio of fragmented DNA relative to total DNA was determined up to 4 h after the start of incubation (Fig. 2B). The ratio of fragmented DNA rapidly increased during the initial 60 min, and then gradually increased up to 4 h.

We have previously reported that a mouse jejunum incubated in Ussing chambers for 2 h exhibited villus damage, while the crypt remained morphologically intact.[10] We found in the present study that the villus damage was evident even in tissue incubated for as short a time as 1 h (Fig. 3). In the villous region, some cells in the epithelial lining exhibited nuclear pyknosis and peripheral chromatin aggregation under the nuclear membrane (Fig. 3E), which are characteristic morphological features of apoptosis. Such features were not apparent in the crypt cells (Fig. 3F).

We next performed the TUNEL assay to identify the type of cells that had undergone DNA fragmentation (Fig. 4). TUNEL-positive nuclei were virtually absent in the mucosa just after the start of incubation. However, in the mucosa incubated for 1 h, cells with TUNEL-positive nuclei were frequently identified among the villous epithelial cells (enterocytes), while they were hardly seen among the crypt epithelial cells. The number of enterocytes with TUNEL-positive nuclei gradually increased after the start of incubation, the increase being statistically significant after 60 min of incubation (Fig. 4 G).

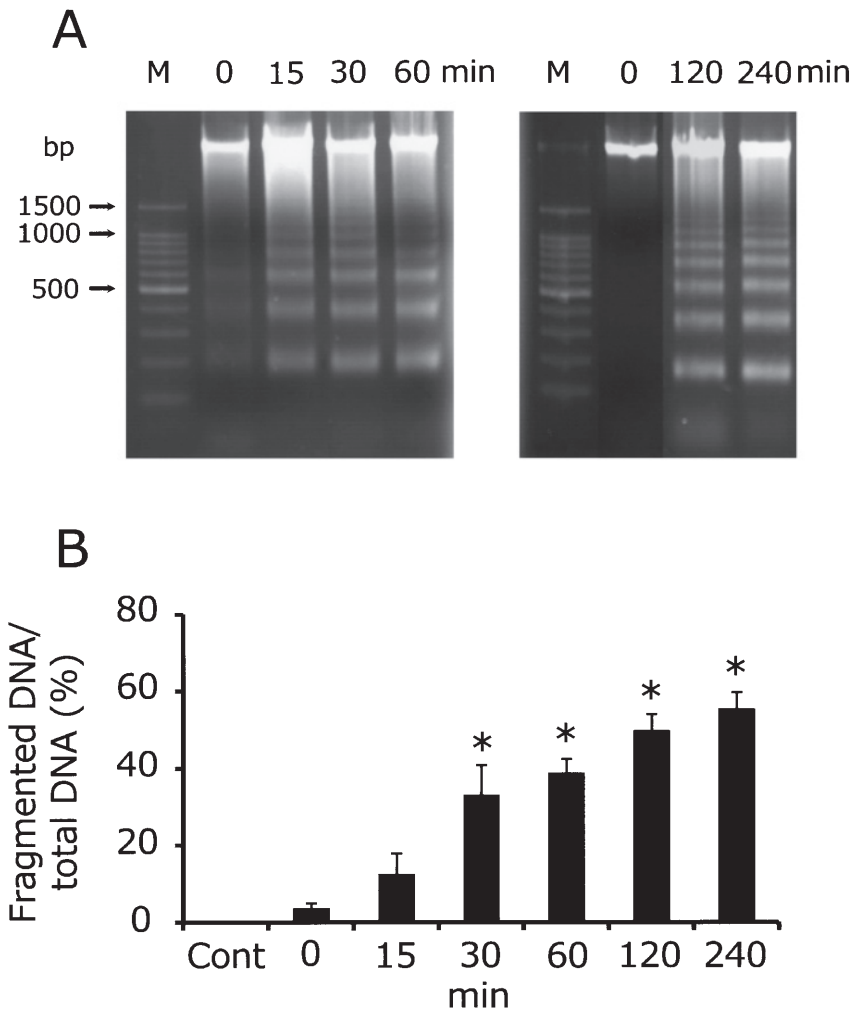

Fig. 2 DNA laddering assay. A) Representative agarose gels of DNA (20 μg) extracted from jejunum mucosa incubated in Ussing chambers for indicated periods (in min). One animal was used for each panel. 0 min, just after start of incubation. M, 100-bp DNA size markers. B) Time-course characteristics of DNA fragmentation. Percentage of fragmented DNA (< 1500-bp) relative to total DNA is shown. Control (Cont) is DNA in mucosa obtained from a freshly isolated jejunum. *p<0.05 compared with 0 min (n=4–26).

DISCUSSION

Our results, taken together, suggest that apoptotic cell death was involved in the villous injury to the mouse jejunum incubated in an Ussing chamber. The apoptotic features were hardly apparent just after the start of incubation, but became evident within 60 min. Apoptosis as detected by TUNEL staining was evident in the enterocytes, although scarcely detectable in crypt epithelial cells, at least within 1 h of incubation. The mechanism for initiating and executing apoptosis under this condition, however, remains unknown.

There are several animal models for intestinal injury in which apoptosis is probably the major mode of epithelial cell death. Injuries induced by pneumonia-induced sepsis, by a variety of

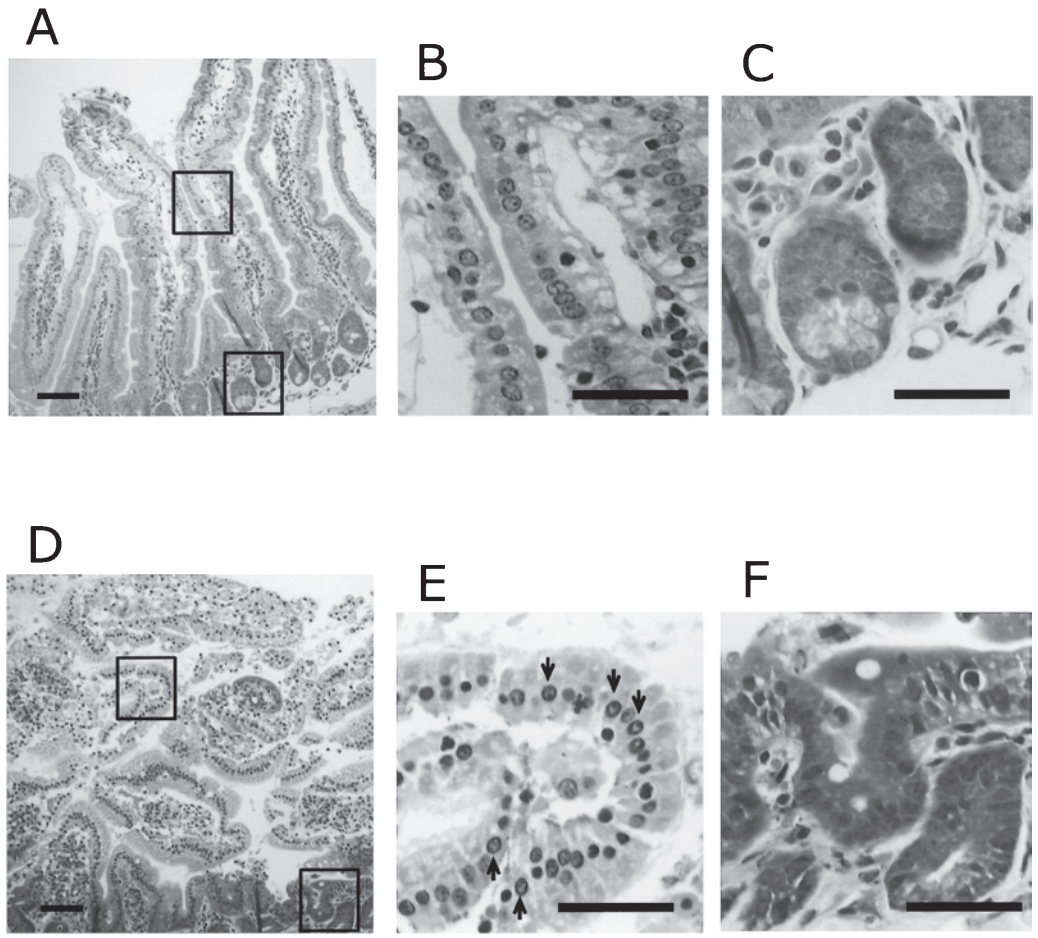

Fig. 3 Histology of jejunum mucosa incubated in Ussing chambers for 0 h (A, B and C) and 1 h (D, E and F). Hematoxylin and eosin staining. B and E show villus region, and C and F show crypt region, and represent an enlargement of enclosed areas in panels A and D, respectively. Arrows indicate morphological features of apoptosis. Data are representative of 3 independent experiments with similar results. Bars are 50 μm for A and D, and 25 μm for B, C, E and F.

drugs, mutagens, and ionizing radiation or by injection of the monoclonal anti-CD3 antibody have been demonstrated to occur mainly in the crypt region.[12-16] On the other hand, the epithelial damage and apoptosis induced by ischemia-reperfusion, traumatic brain injury and fasting occur mainly in the villus region.[5-6, 8, 17] Since the present preparation in Ussing chambers exhibits both the villus damage and apoptosis, it can be used as an *in vitro* model for studying intestinal injuries by ischemia-reperfusion, traumatic brain injury and fasting. Many factors such as reactive oxygen or nitrogen species, TNF-α and the platelet-activating factor have been implicated in these diseases with villous epithelial apoptosis.[7, 18-21] In addition, a loss of cell anchorage to the extracellular matrix often induces a specific type of apoptosis known as detachment-induced cell death or anoikis.[22-24] Therefore, it would be interesting to examine whether these factors might be responsible for induction of the mucosal injury and apoptosis observed in the mouse jejunum incubated in Ussing chambers.

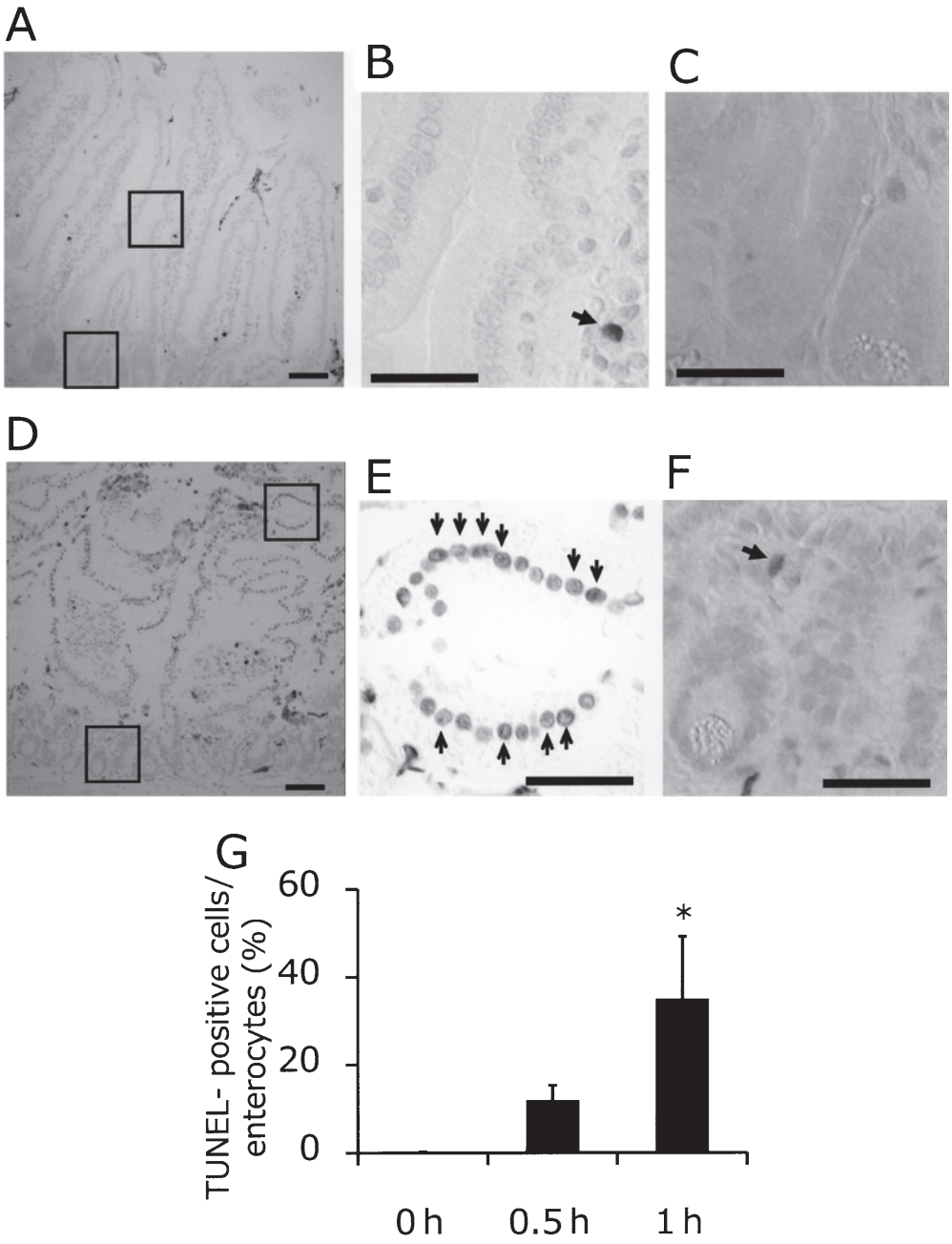

Fig. 4 Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) analysis. Staining of jejunum mucosa incubated in Ussing chamber for 0 min (A, B and C) and 60 min (D, E and F). B and E show villus region, and C and F show crypt region, which represent an enlargement of enclosed areas in panels A and B, respectively. Bars are 50 μm for A and D, and 25 μm for B, C, E and F. Arrows indicate positive staining of villous epithelial cells (enterocytes). Arrowheads indicate positive staining of subepithelial cells. G) Number of TUNEL-positive enterocytes in mucosa incubated for 0 min, 30 min and 60 min. Approximately 100 nuclei in epithelia-like cell lining in villous region were evaluated in each preparation. Number at 60 min was significantly higher than that at 0 min (n=3–4).

In summary, apoptotic death of enterocytes was involved in the injury to villi when the proximal mouse intestine was incubated in Ussing chambers. Further elucidation of the mechanism underlying the villous damage in this injury model would shed light on a variety of mucosal disorders such as inflammatory bowel diseases, and also providing a clue to reducing graft preservation injury for intestinal transplantation.

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