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Transmissible gastroenteritis virus induced apoptosis in swine testes cell cultures

Brief Report

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Summary. Transmissible gastroenteritis virus (TGEV) is a coronavirus which causes severe gastroenteritis and atrophy of intestinal villous epithelial cells in piglets. However, the mechanism of cell death caused by TGEV is not known. In this study, we report that TGEV induces cell death by apoptosis. TGEV-induced apoptosis was demonstrated by agarose gel electrophoresis, electron microscopy, and terminal deoxytransferase digoxigenin-dUTP nick end labeling (TUNEL). Double labeling experiment confirmed the result from electron microscopy and showed that most of the apoptotic cells were bystander cells as they were negative for TGEV nucleic acids. Results of this study indicate that TGEV induces apoptosis in vitro and that most of the cells undergoing apoptosis are bystander cells, thus amplifying the cytopathic effect of TGEV.

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Transmissible gastroenteritis virus (TGEV), a coronavirus, is a pleomorphic enveloped virus with a positive-sense single-stranded RNA genome [31]. TGEV is associated with an economically important disease, transmissible gastroenteritis (TGE), and causes severe vomiting, diarrhea, and high mortality in piglets during the first few weeks of life [31]. TGE has been recognized since 1946 [6] and has been extensively studied. However, the mechanism of TGEV-induced cell death is still unclear. Recently, several DNA [9, 23, 27, 29, 30] and RNA viruses [8, 10, 12, 18, 26, 32, 36, 41, 46] have been shown to induce cell death by apoptosis. We investigated whether TGEV kills cells by apoptosis, if there is a

correlation between cytopathic effect (CPE) and apoptosis, and whether infected, bystander or both infected and bystander cells undergo apoptosis.

The virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, IA), referred to as CHV, was used to inoculate swine testes (ST) cells on 6-well plates (Corning Costar Corporation, Oneonta, NY) or 8-well chamber slides (Nunc, Naperville, IL) at a multiplicity of infection of 0.01. Uninoculated and inoculated cells on 6-well plates were observed for cytopathic effect (CPE) and at 12, 24, 32, 40, and 48 h postinoculation (h p.i.). The cells were then scraped with a cell scraper (Fisher, PA), transferred to 1.5 ml microfuge tube, and centrifuged at 1100 $\times$ **g** for 3 min. Cells were washed, and resuspended in 200 μl of cold phosphate buffer saline (PBS) pH 7.2. Total cell DNA was obtained using a Qiagen tissue kit (Qiagen, CA). DNA was precipitated and resuspended in 60 μl of TE buffer. Finally, 10 μl of the total 60 μl cell DNA was electrophoresed on a 1.2% agarose gel (Amresco, Solon, OH). DNA bands were stained with ethidium bromide, visualized by UV illuminator, and recorded on Polaroid type 57 film (Polaroid Corporation, Cambridge, MA). This assay was repeated three times using 3 different sets of cells. Cytopathic effect (CPE) characterized by rounding and sloughing of cells was detected at 24 h p.i. in TGEV-inoculated ST cell cultures. The extent of CPE increased by 48 h p.i. (Table 1). Apoptosis was observed at 24 h p.i. and was characterized by fragmentation of DNA to approximately 180 base pair ladder. Both CPE and relative amounts of low molecular weight (LMW) DNA were evident at 24 h p.i. and peaked at 48 h p.i. (Table 1 and Fig. 1).

For electron microscopy study, uninoculated and TGEV-inoculated cells on 6-well plates were washed twice with cold phosphate buffer saline (PBS) pH 7.2, fixed with 3% glutaraldehyde for 3 min, and harvested by scraping at 12, 24, 32, 40, and 48 h p.i. Media from uninoculated and TGEV-inoculated wells at

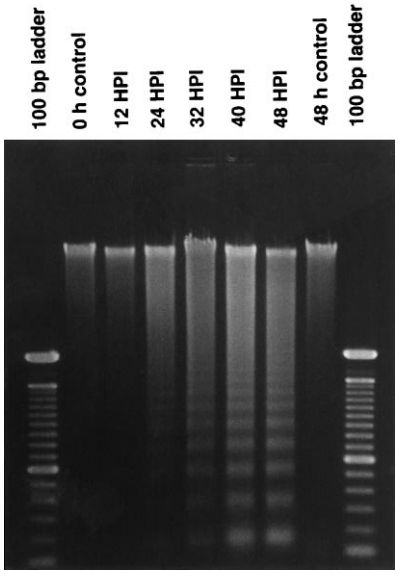


Fig. 1. Analysis of DNA fragmentation as an evidence of apoptosis by agarose gel electrophoresis. A 10 μl of total DNA extracted from TGEV-inoculated or uninoculated ST cell cultures was loaded on to each lane of a 1.2% agarose gel. A DNA ladder of bands in multiplication of about 180 nucleotides in size was detected in the DNA of TGEV-inoculated cell culture at 24 h p.i. with highest concentration at 48 h p.i.

Table 1. Progression of cytopathic effect and concentration of low molecular weight DNA in TGEV-inoculated ST cell cultures at 12, 24, 32, 40, and 48 h postinoculation[a]

Measurement	Hours				
	12	24	32	40	48
% CPE	0	2	10	25	50
Relative amount of LMW DNA[b]	−	±	+	++	+++

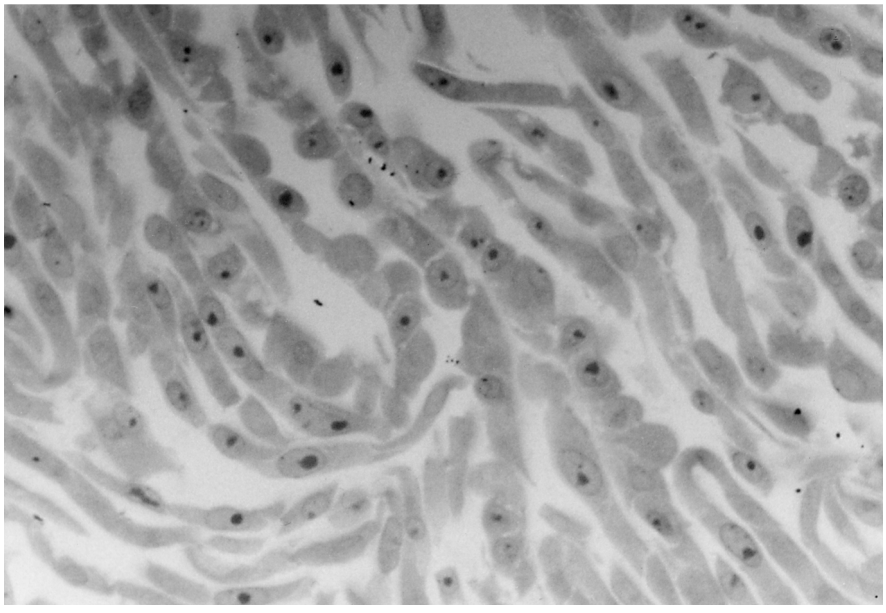
[a]Progression of CPE and amount of LMW DNA was done by using 3 replications of uninoculated and inoculated ST cells culture

[b]Relative amount of LMW DNA was determined by comparing the intensity of the DNA bands in agarose gels as follows: − = no band, ± = very weak, + = weak, ++ = moderate, +++ = strong

each time point was collected and centrifuged to collect the detached cells. The pellets of detached cells were fixed, resuspended, and mixed with suspension of the adherent cells at each time point. Cells were pelleted at 6000 × **g** for 2 min at 4 °C. The pellets were washed three times with 0.1 M sodium cacodylate buffer (pH 7.2) for 15 min each, post fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at room temperature, dehydrated in an acetone series, and embedded in plastic [11]. All cells from at least two sections of TGEV-inoculated and uninoculated cells at each time point were examined by electron microscopy (Hitachi H500). Examination of thick sections of TGEV-inoculated ST cells revealed several cells with condensation and margination of nuclear chromatin (Fig. 2B). In contrast, these changes were not observed in uninoculated ST cells (Fig. 2A). Ultrastructural changes characteristic of cell lysis and apoptosis were detected in TGEV-infected cells. Lytic cells were characterized by disrupted nuclear and cytoplasmic membranes, and degenerate cell organelles (Fig. 3B). There were several cells undergoing lysis, and most of these cells had several viral particles budding from the endoplasmic reticulum (Fig. 3B). Apoptotic cells were characterized by condensation and sharp margination of nuclear chromatin, formation of large, electrolucent vacuoles in the cytoplasm, intact cell organelles, and intact nuclear and cytoplasmic membranes (Fig. 3C). Viral particles were not detected in apoptotic cells. In contrast, cells in the uninoculated wells were normal (Fig. 3A).

In situ hybridization (ISH) was performed before in situ detection of apoptosis. Fluorescein-labeled RNA probe was prepared from plasmid pPSP.FP_1 as reported previously [34] which contains the TGEV S gene segment from nucleotides 1 678 to 2 250 [3, 42]. Specificity of the probe for TGEV was determined by dot blot hybridization [43]. Briefly, uninoculated and TGEV-inoculated ST cells on 8-well chamber slides at 12, 24, 32, 40, and 48 h p.i. were used

A



B

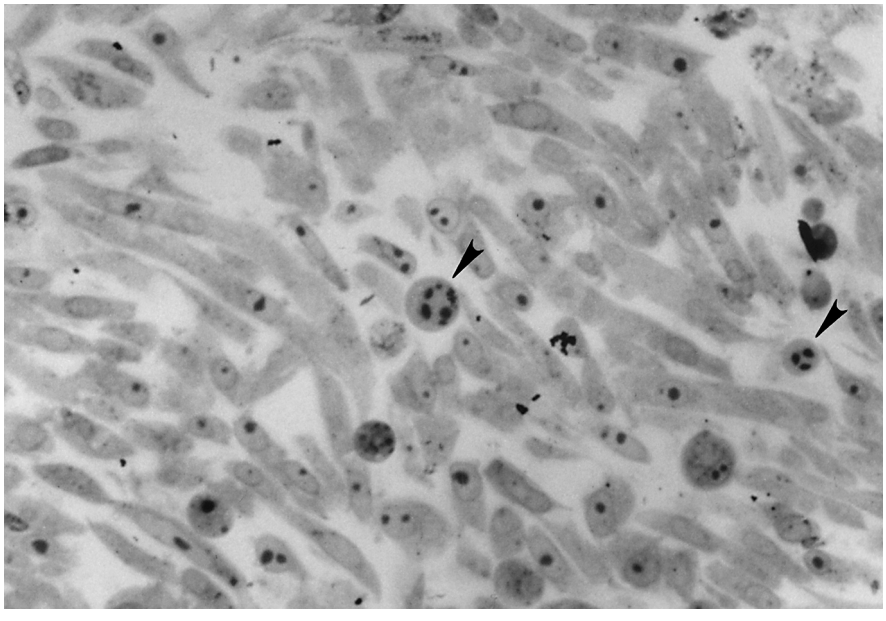


Fig. 2. Thick sections of uninoculated (**A**) and TGEV-inoculated ST cell cultures (**B**) at 24 h p.i. The section of TGEV-inoculated ST cell culture showed sharp condensation and margination of nuclear chromatin along the nuclear membrane of the apoptotic cells (arrowhead)

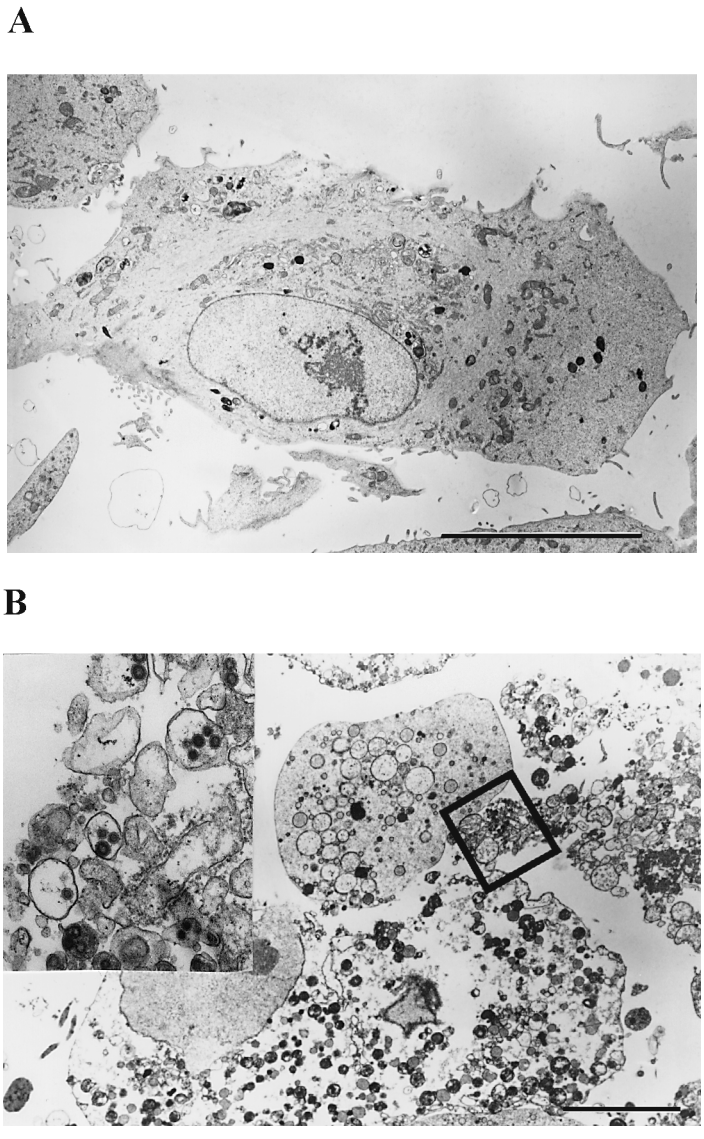


Fig. 3 (*continued*)

for in situ hybridization (ISH) as described previously [35]. Uninoculatd and TGEV-inoculated cells were treated with proteinase K (1 μg/ml) at 37 °C for 10 min, washed, and incubated with 20% cold acetic acid for 15 sec. Uninoculated and TGEV-inoculated cells were hybridized with 50 μl of hybridization buffer containing 2 μl of fluorescein-labeled RNA probe (1 ng/μl). Uninoculated and TGEV-inoculated cells were treated with RNase A(20 μg/ml) at 37 °C for 30 min, and washed in series of SSC. The hybridization signal was detected by incubating with either anti-fluorescein peroxidase antibody (1:50) or anti-fluorescein alkaline phosphatase antibody (1:400) (Boehringer Mannheim, Indianapolis, IN) for 1 h at room temperature. Slides were incubated with DAB or NBT and BCIP and counterstained with either hematoxylin or nuclear fast red. Con-

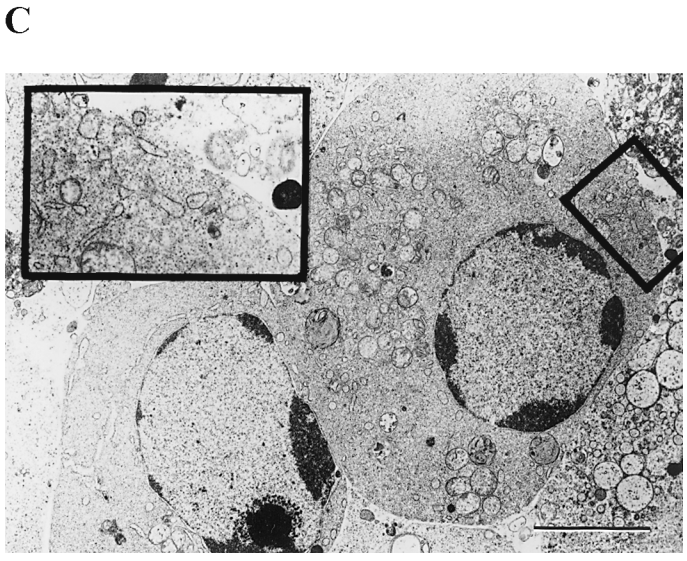


Fig. 3. Electron micrograph of uninoculated (**A**) and TGEV-inoculated ST cell culture (**B**, **C**) at 32 h p.i. Bars: 10 μm (**A**), 5 μm (**B**), and 2.5 μm (**C**). The virus inoculated cells in TGEV-inoculated ST cell culture (**B**) had several virus particles in vesicles of the endoplasmic reticulum (inset) and were undergoing cell lysis. In contrast, the uninfected cells in TGEV-inoculated ST cell culture (**C**) had no virus particles in the endoplasmic reticulum (inset) and exhibited shrinking of cells and nuclei, condensation of nuclear chromatin, and intact cell and nuclear membranes

trols included uninoculated ST cells, RNase-treated cells inoculated with TGEV, TGEV-inoculated cells hybridized with positive-sense fluorescein-labeled RNA probe, and TGEV-inoculated ST cells hybridized with hybridization buffer free of probe. The cells in the TGEV-inoculated wells were positive for TGEV and cells in the uninoculated wells were negative. The TGEV positive cells had brown color localized in the cytoplasm without any color in the nucleus (Fig. 4B). In contrast, cells in the uninoculated wells were negative (Fig. 4A).

Cells on 8-well chamber slides were counted to determine cell viability at each time point. Both the adherent and detached cells were collected, centrifuged, resuspended in PBS (pH 7.2), and stained with trypan blue to determine cell viability [33]. Cell viability was determined in triplicates. The viability count results showed that the percentage of live cells decreased after 24 h p.i. and was lowest at 48 h p.i. (Fig. 5A). The adherent cells on 8-well chamber slides were used to detect apoptosis in situ. The TUNEL method was performed using an ApopTag kit (Oncor, Gaitherburg, MD) according to manufacturer's instructions with slight modifications. Briefly, 8-well chamber slides were subjected to the enzymatic incorporation of digoxigenin-labeled nucleotide with enzyme terminal deoxynucleotidyl transferase, washed and incubated with either anti-digoxigenin peroxidase and 3,3′-diaminobenzidine (DAB) or (1:400) anti-digoxigenin alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and 45 μl of 4-nitroblue tetrazolium (NBT), and 35 μl of 5-bromo-4-chloro-3-indolyl-phosophate (BCIP) in detection

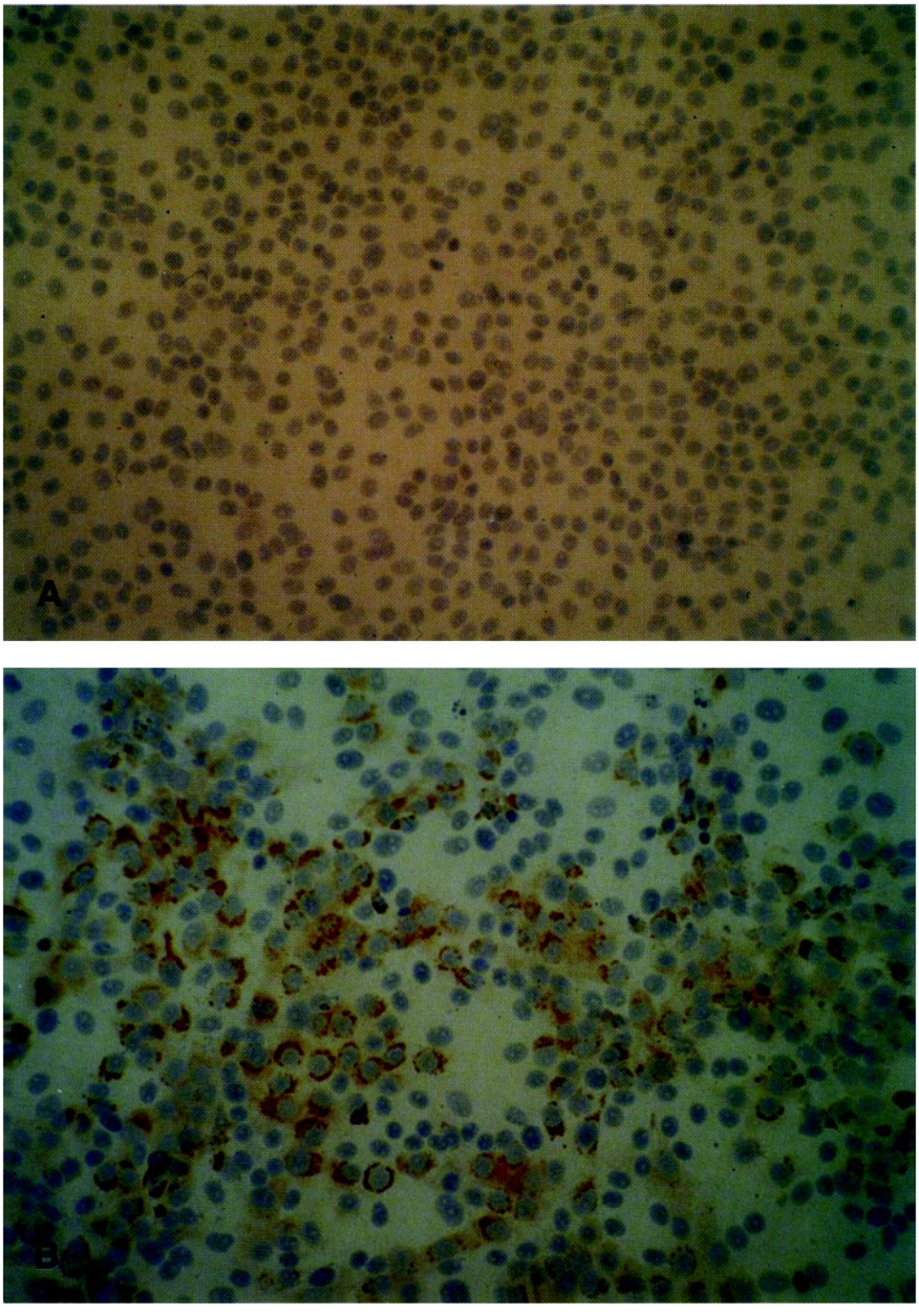


Fig. 4. In situ hybridization of uninoculated (**A**) and TGEV-inoculated ST cell culture (**B**) at 32 h p.i. with fluorescein-labeled RNA probe produced from pPSP.FP$_1$ (20 $\times$ magnification). Infected ST cells exhibited dark brown color specifically localized in the cytoplasm but uninoculated cells were negative

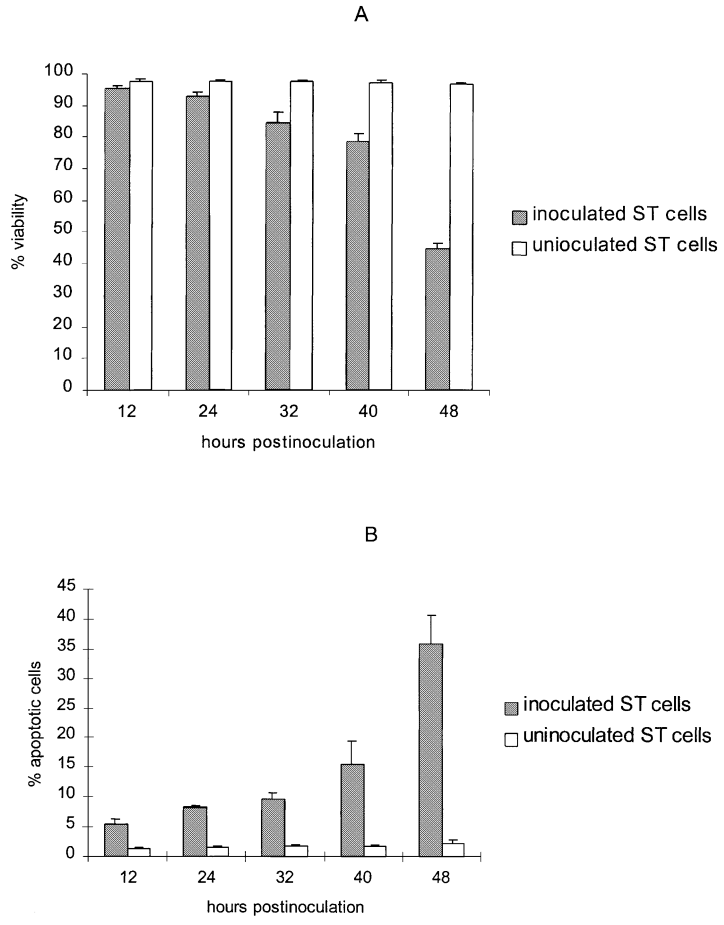


Fig. 5. Relative frequency of viable (**A**) and apoptotic cells (**B**) in uninoculated and TGEV-inoculated ST cell culture. The cell viability decreased with time in TGEV-inoculated ST cell cultures and was lower than that in uninoculated ST cell cultures at each time point (**A**). In contrast, apoptotic cells increased with time in TGEV-inoculated ST cell cultures and was higher than that in uninoculated ST cell cultures at each time point (**B**)

buffer. The slides were counterstained with either nuclear fast red or hematoxylin. The cells with the signal localized within the nucleus without diffuse staining in the cytoplasm or extracellular area were interpreted as being apoptotic cells [5]. The number of apoptotic cells was determined by counting 1 000 cells in each of 5 different areas in each well of the 8-well chamber slides. Cells were counted in four separate wells and the number of apoptotic cells determined by counting 5 000 cells. The mean percentage of apoptotic cells was determined using the percentage of apoptotic cells in 20 000 cells counted in four inoculated, and four uninoculated wells. The results are shown as the mean percentage of apoptotic cells, and one standard deviation from the mean. TUNEL-positive cells in TGEV-

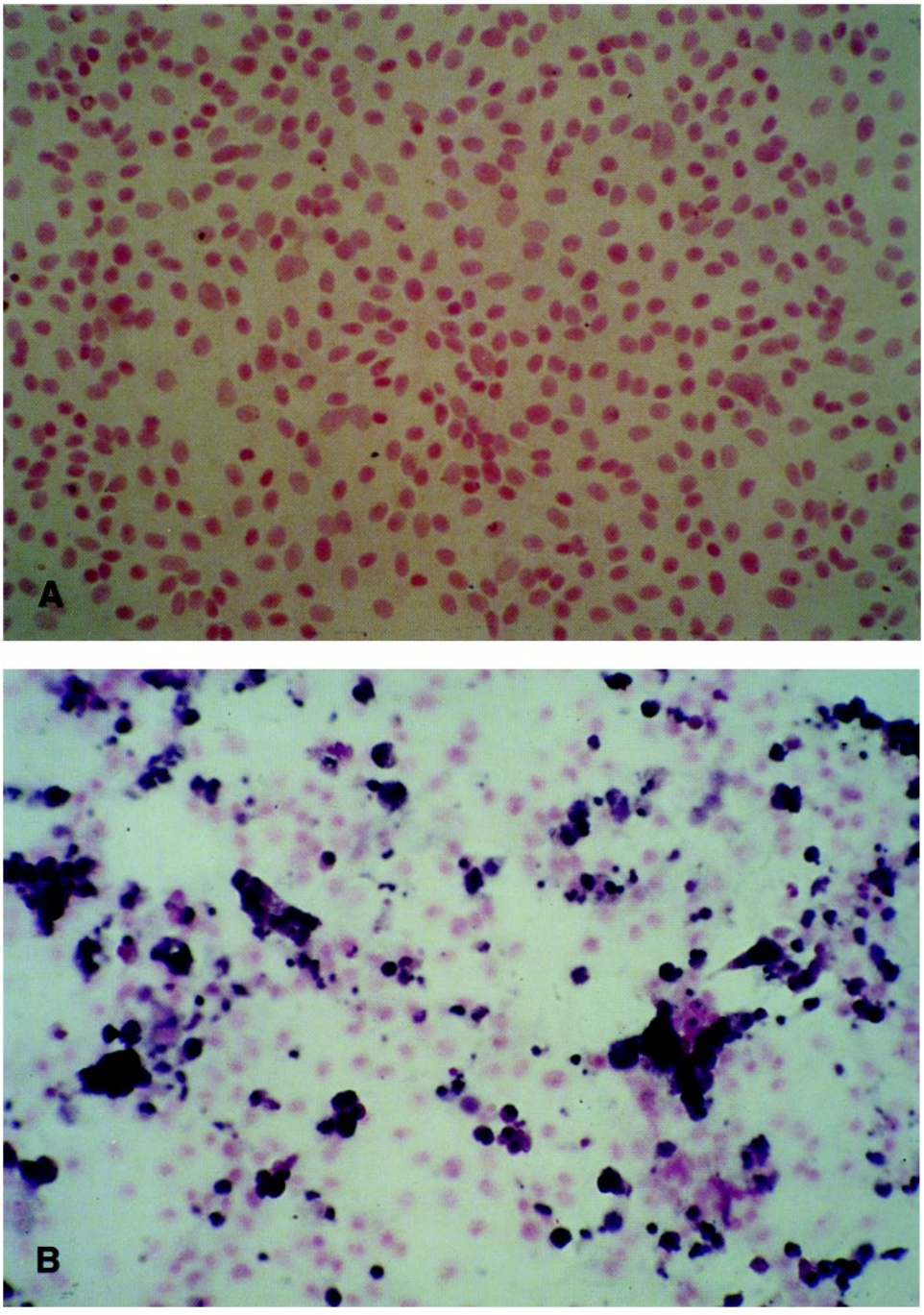


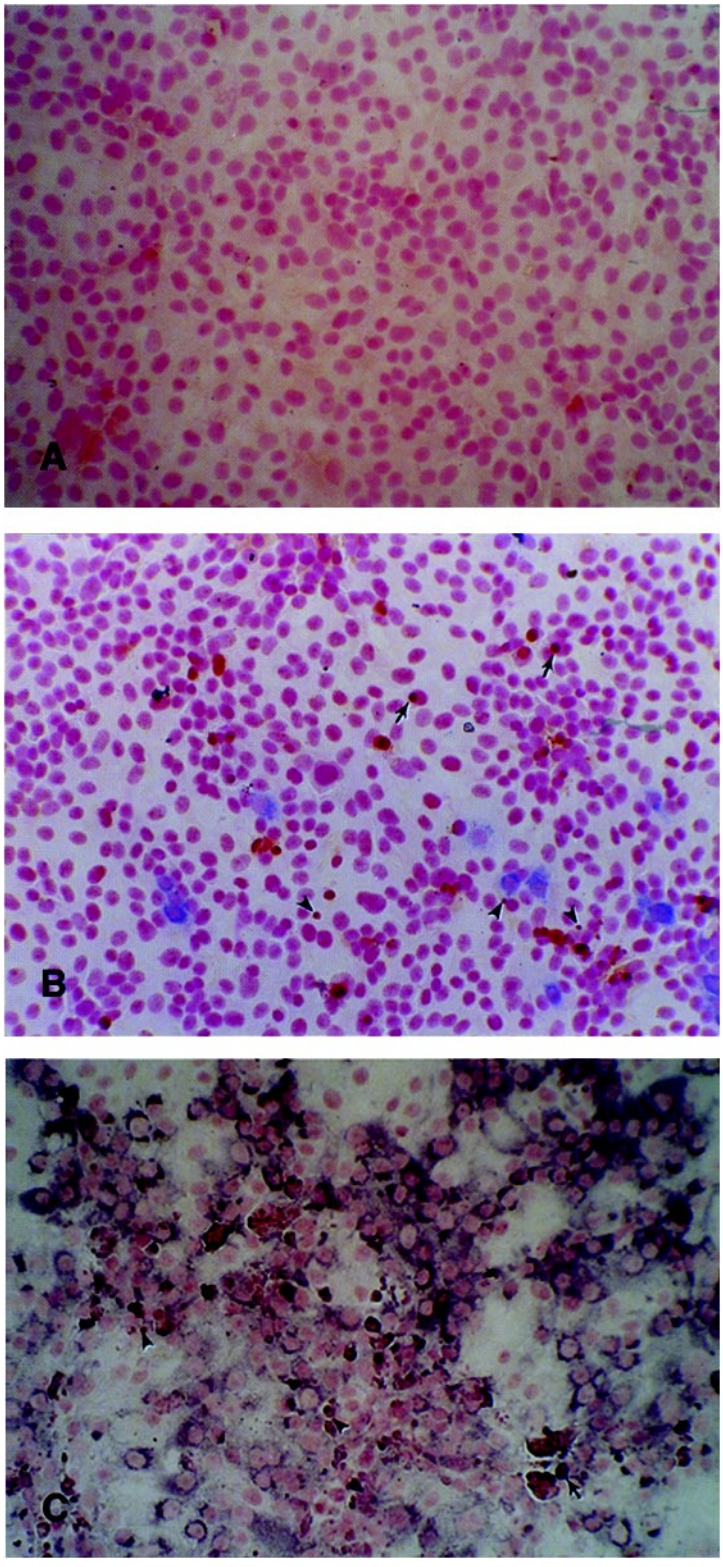
Fig. 6. TUNEL analysis for the detection of apoptosis in uninoculated (**A**) and TGEV-inoculated ST cell culture (**B**) at 40 h p.i. (20 × magnification). Several apoptotic cells were observed in TGEV-inoculated cell culture with dark purple staining localized in the nucleus

inoculated wells were detected as early as 12 h p.i. and their number peaked at 48 h p.i. There were a higher percentage of apoptotic cells in the inoculated wells than that in the uninoculated wells at each time point, and the percentage of positive cells increased with time (Fig. 5B). The apoptotic cells had purple nuclei and no cytoplasmic staining (Fig. 6B). Several apoptotic bodies were blebbing out of the cytoplasm and were scattered in the intercellular space. There were a few TUNEL-positive cells in the uninoculated wells (Fig. 6A).

In order to determine TGEV-induced apoptosis in bystander cells, uninoculated and TGEV-inoculated ST cells on 8-well chamber slides were used at 12, 24, 32, 40, and 48 h p.i. for ISH followed by TUNEL labeling. Media from 8-well chamber slides at each time point was also collected and centrifuged for obtaining the detached cells to perform double-labeling technique. TGEV-infected cells in TGEV-inoculated wells had purple cytoplasm whereas apoptotic cells had brown nuclei (Fig. 7B and 7C). In contrast, the ST cells in uninoculated wells had neither purple cytoplasm nor brown nucleus (Fig. 7A). Both the TGEV-positive and apoptotic cells were detected as early as 12 h p.i. (Fig. 7B) and their numbers increased with time. The majority of the adherent cells were positive for either TGEV nucleic acid, or apoptosis. Most of the apoptotic cells lacked TGEV RNA whereas most of the TGEV-positive cells did not exhibit apoptosis. At 48 h p.i. there were, however, a few double positive cells which were positive for TGEV RNA and had round TUNEL-positive particles in the cytoplasm (Fig. 7C). These particles were apoptotic bodies that were phagocytized from the apoptotic cells rather than nuclei of the double positive cells. About 75–80% of the detached cells at 24, 32, 40, and 48 h p.i. were positive for either TGEV nucleic acid or apoptosis. Another 20–25% of the detached cells at these time points were double positive cells. Most of these cells appeared to be necrotic cells as these cells were swollen, lacked nucleus, and had diffuse TUNEL signal. However, there were about 2–5% of the necrotic double-positive detached cells that had discrete staining of apoptotic bodies. These infected cells may phagocytize apoptotic bodies from the neighbor cells.

This is the first report showing that TGEV is capable of inducing apoptosis. This was demonstrated by several methods including analysis of total cellular DNA for low molecular weight DNA by agarose gel electrophoresis, TUNEL labeling for detection of DNA fragmentation in situ, and transmission electron microscopy for nuclear morphology. There was general agreement in results obtained with different techniques. In the analysis of total cellular DNA, DNA

Fig. 7. Double labeling analysis of viral RNA and apoptotic cells in uninoculated (**A**) and TGEV-inoculated ST cell culture at 12 h p.i. (**B**) and 40 h p.i. (**C**) (20 × magnification). Cells with purple color cytoplasm were positive for TGEV RNA and were detected in TGEV-inoculated ST cell culture only. Cells with dark brown nuclei (arrows) by TUNEL labeling represented apoptotic cells and several apoptotic bodies (arrowheads) were scattered throughout the slides. However, there were no cells that were positive for both TGEV RNA and apoptosis


A
B
C

fragmentation bands were detected at 24 h p.i. which peaked at 48 h p.i in the TGEV-inoculated cells. The DNA fragmentation pattern in multiplication of 180–200 base pairs strongly supports the presence of an apoptosis process in the cell culture [39]. There was a correlation between appearance of apoptosis and CPE that began at 24 h p.i. and peaked at 48 h p.i.

Using electron microscopy, we found several cells in TGEV-inoculated wells having sharp nuclear chromatin condensation along the nuclear membrane with intact nuclear and cell membranes. These are considered the characteristic of apoptosis [14]. Viral particles were present in lytic cells but not in the apoptotic cells, suggesting that infected cells were undergoing cell lysis. McClurkin and Norman [25] also reported changes in TGEV-inoculated cells suggestive of apoptosis, although the characteristics of apoptosis were not recognized at that time. They observed changes in the nuclei of the ST and swine kidney cell cultures infected with several isolates of TGEV stained with acridine orange which were characterized by shrunken, marked clumping and margination of the chromatin material along the nuclear membrane [25].

By using TUNEL labeling, we were able to identify apoptotic cells in the TGEV-inoculated cell culture. The number of apoptotic cells increased with time and peaked at 48 h p.i. However, TUNEL labeling is believed to be more sensitive than a DNA fragmentation assay as it can detect apoptotic cells earlier [7]. Both the individual and syncytial cells were positive by TUNEL. We performed a double-labeling experiment to determine whether infected or bystander cells were the primary cells undergoing apoptosis. Surprisingly, the uninfected bystander cells were undergoing apoptosis but infected cells were not. These results were also confirmed by electron microscopy.

The mechanism of TGEV-induced apoptosis in bystander cells is not known. Two coronaviruses, mouse hepatitis virus and feline infectious peritonitis virus, have also been shown to induce apoptosis in bystander cells [8, 10]. Several cytokines, such as tumor necrosis factor-α (TNF-α) [13, 15, 47] and transforming growth factor-β (TGF-β) [40, 45] have been shown to promote apoptosis in several cells. Interferon (IFN)-γ and TNF-α [28, 38] have been shown to induce apoptosis by upregulating Fas antigen expression. It is known that rat testicular cells are highly capable of expression Fas ligand [37]. The testicular cell lines from mice have also been shown to be capable of expressing Fas antigen and can be upregulated by IFN-γ [21]. The rat testicular cells are capable of expressing both interferon-α and -γ [4].

TGEV has been shown to be a good IFN-α inducer [17, 19] which inhibits TGEV replication in vitro but has little or no effect in vivo [17, 22]. IFN-α also has cytotoxicity effect for both porcine intestinal explant and porcine cell lines (ST, PK-15 porcine kidney and intestinal epithelial cells) either infected or uninfected with TGEV [2, 20, 44]. Therefore, the antiviral activity of IFN-α against TGEV may partly be due to cytotoxicity effect [44]. Furthermore, cytopathic strain of bovine viral diarrhea virus can prime uninfected cells to undergo apoptosis by type I IFN[1]. IFN-α has been shown to upregulate dsRNA-activable protein kinase PKR that is a tumor suppressor gene by activating apoptosis [24]. It has also been

proposed that the mechanisms responsible for IFN-α inducing TGEV-infected ST cells destruction may contribute to the pathogenesis of TGEV infection in vivo [2]. Therefore, IFN-α might play a role in inducing apoptosis in bystander cells and thus amplifying the cytopathic effect of TGEV.

At this time, we do not know if IFN-α, other cytokines, some soluble factors released from infected cells or viral products are responsible for inducing apoptosis in bystander cells. In this study, we have shown that TGEV induces apoptosis in ST cell culture, and infected cells undergo cell lysis. Most of the apoptotic cells were bystander cells whereas most of the productively infected cells did not undergo apoptosis. However, whether TGEV is capable of inducing apoptosis in enterocytes in vivo is still unresolved.

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