Immunohistochemical detection of caspase 3 and proliferating cell nuclear antigen in the intestines of dogs naturally infected with parvovirus

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Abstract

Canine parvovirus (CPV) causes a contagious and fatal viral disease in dogs characterized by hemorrhagic enteritis. Apoptosis is a programmed cell death and one of the primary markers of this process is caspase 3. Proliferating cell nuclear antigen (PCNA) is also associated with important vital cellular processes. This study was conducted to examine the expressions of caspase 3 and PCNA in the intestinal samples of dogs naturally infected with CPV using immunohistochemical methods. A total of 30 dogs with parvoviral enteritis and five control dogs gut tissues were evaluated for caspase 3 and PCNA expressions. Increased immunoactivities of caspase 3 and PCNA were observed in epithelial, crypt and inflammatory cells in the CPV-infected dogs. Increased expressions of both markers were observed being related to the severity of disease. These results demonstrated the important roles of caspase 3 and PCNA in CPV pathogenesis. These markers may be useful for early diagnosis, estimation of the severity or future treatment strategies of this important disease.

Introduction

Canine parvovirus (CPV) infection is an important viral disease of dogs. Its usual clinical form is parvoviral enteritis; but, it also manifests itself as a parvoviral myocarditis or mixed form. Young dogs, aged between 6 and 20 weeks, are the most susceptible ones to parvoviral enteritis. Clinically infected dogs become anorectic and lethargic and may vomit and develop diarrhea, with transient pyrexia occurring commonly. Apoptosis is an evolutionarily conserved process of programmed cell death (PCD) or a highly regulated cell suicide mechanism. Cells dying through PCD often undergo distinct morphological changes known as apoptosis and cleave their deoxyribonucleic acid (DNA) into small fragments. The caspase family of cellular proteases initiates and executes apoptotic cell death. Caspase 3, a pivotal effector caspase, is an essential protease of the apoptotic process. Proliferating cell nuclear antigen (PCNA) is an intranuclear 36.00-kD non-histone protein and one of the central molecules responsible for decisions regarding life and death of the cell. The PCNA immunostaining characteristics allow the identification of cells in different phases of the cycle. The expression of PCNA increases during the G1-phase, peaks at the S-phase and declines during G2/M-phases of the cell cycle. This protein has also an essential role in nucleic acid metabolism as a component of DNA replication and repair mechanisms. An increase in PCNA expression levels may be induced by growth factors or as a result of DNA damage in the absence of cell cyclin.

The CPV infection is an important and fatal disease characterized by vomiting, hemorrhagic enteritis and intestinal findings. However, the pathogenetic pathways of the disease are not completely understood. Therefore, this study was conducted to examine the immunohistochemical expressions of caspase 3 and PCNA in the intestines of dogs naturally infected with parvovirus.

Materials and Methods

In this study, 30 intestinal samples from dogs with positive parvovirus rapid tests or suspicious diagnoses
were collected from the archive of the Department of Pathology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Turkey. The dogs examined in this study were aged 2 to 5 months and were of both sexes and different breeds. Necropsy notes were evaluated and dogs with enteric parasitic infections were excluded from the study. Intestinal tissues of five puppies of similar ages died due to traffic or other accidents were used as controls. Ethical approval was not required for this retrospective study.

Four serial sections were taken from the paraffin blocks of intestinal samples of 30 infected dogs and five control dogs. One of the sections was stained with Hematoxylin and Eosin (H&E). Intestinal samples taken from duodenum, jejunum, ileum, cecum, colon and rectum, especially the ileocecal region, of dogs with suspected parvoviral enteritis were selected for this study.

The remaining three sections were immunostained with CPV (mouse monoclonal anti-parovirus antibody (CPV1-2A1; Abcam, Cambridge, UK), caspase 3 (rabbit polyclonal anti-caspase 3 antibody; Abcam) and PCNA (rabbit polyclonal anti-PCNA antibody (Abcam) antibodies according to the manufacturer’s instructions using a routine streptavidin–biotin peroxidase method (SBPM). Morphometric evaluation was performed using the Database Manual cellSens Life Science Imaging Software System (Olympus Corp., Tokyo, Japan).

To determine the percentage of immunostained cells for each marker, 100 cells were counted in 10 fields on each section using a 40× objective for all groups. The percentage of immunostained cells in each sample was calculated and statistical analysis was performed.

The SPSS Software (version 20.0; IBM Corp., Armonk, USA) was used for analysis of immunohistochemistry results. The variables were assessed by Duncan test and ANOVA tests were used to compare groups. Values for \( p < 0.05 \) were considered statistically significant.

Results

According to necropsy notes, none of the puppies were vaccinated for parvoviral enteritis. The puppies had inappetence, depression, bloody diarrhea and vomiting. They died 3-4 days after the initial symptoms. According to the clinical symptoms obtained from necropsy notes, dogs were classified based on the disease severity. In this study, 3 mild, 10 moderate and 13 severe cases were evaluated. No sex predisposition was observed.

Examination of the archived notes revealed marked dehydration and weakness as common findings and the lesions were primarily localized in the small intestine (24 out of 30 cases). The lesions were segmental or widespread and irregularly distributed, with frequent findings of intestinal hemorrhage and fibrinous exudate. Malodorous and watery or hemorrhagic contents were the common findings and hemorrhages were marked and typically localized especially in the ileocecal valve (Fig. 1). Erosion and ulcers were also observed in the gut mucosa of severely infected dogs. Intestinal walls were swollen, edematous and hemorrhagic in severe cases. In severely infected dogs, Peyer’s patches were edematous and hemorrhagic and sometimes evident from the serosal or mucosal aspects. Different amounts of fluid were accumulated in the abdominal cavity of 19 cases. Severe hyperemia in the mesenteric vessels and enlargement and hemorrhage were commonly observed in mesenteric lymph nodes.

Histopathological examination of the intestinal sections revealed that all the small and large intestinal sections were infected with the disease; but, the most marked lesions were localized in the ileocecal junction. Serosal edema, desquamation of the villi, erosion, ulcers and hemorrhages of the mucosa were common. The ulcerous areas showed inflammatory cell infiltrations especially composed of neutrophils and a small number of lymphoid cells. Atrophies of the Peyer’s patches or total necrosis were diagnosed in severe cases (Figs. 2A and 2B). Desquamation of the villi and fusion of cryptic epithelial cells or villi were observed in 15 cases. Regenerative crypt cells were characteristic findings in all cases. Although segmental lesions were detected in 17 cases, lesions were localized in the entire intestine, from the duodenum to rectum, in severely infected dogs. Secondary bacterial colonies were noticed in 13 dogs.

A total of 26 cases were positive for parvovirus; for that reason, remained negative four cases removed from caspase 3 and PCNA immunohistochemistry in this study. The CPV immunopositive cases revealed a positive immunoreaction being localized especially in cryptic epithelial and inflammatory cells. The parvovirus-positive
immunoreaction was primarily observed in histologically lesioned areas (Fig. 2C). There was no positive reaction in the antibody-omitted negative control sections and control dogs' intestinal sections.

The PCNA expression was observed in the control group as well; but, it was prominent in the parvovirus-infected gut samples. The PCNA immunolabeling was primarily detected in the nuclei of proliferative cells. The most prominent reaction was observed in regenerative crypt cells and epithelia of the intestinal villi (Figs. 2D and 2E). The PCNA expression was also detected in relatively normal epithelial cells near the lesioned areas. In addition to epithelial cells, some interstitial cells also expressed PCNA. According to anamnesis and necropsy notes, in dogs suffered for a longer time before death, numerous regenerated cells showed a marked PCNA expression compared to those in dogs died within a short time after initial symptoms. Expression scores increased by severity of the disease (Table 1). The negative control sections showed no PCNA expression.

Marked caspase 3 expression was observed in different cells in lesioned areas. Both epithelial and mesenchymal cells such as crypt cells and epithelial cells of the villi, muscle cells and some peripheral nerve cells showed positive reaction. In addition to lesioned areas, cells near the lesions also expressed caspase 3. Some regenerative cells and abnormal cells also exhibited marked positive immunoreaction (Fig. 2F). Marked expressions were observed in severely affected puppies (Table 1). There was no reaction in the negative control sections (Table 1). A slight expression was observed in the intestinal samples of control dogs. The most common expression for each marker was noticed in epithelial cells. Statistical analysis results of the positive cell percentage were shown in Figure 3.

Table 1. Dog numbers and scoring of the immunohistochemical expression of proliferating cell nuclear antigen (PCNA) and caspase 3 in 26 parvovirus positive cases.

| Method of staining | Number of affected dogs |
|--------------------|-------------------------|
|                    | - | + | ++ | +++ |
| **PCNA expressions** |   |   |    |     |
| Control            | 0 | 4 | 1  | 0   |
| Mild               | 0 | 0 | 2  | 1   |
| Moderate           | 0 | 0 | 5  | 5   |
| Severe             | 0 | 0 | 4  | 9   |
| **Caspase 3 expressions** |   |   |    |     |
| Control            | 0 | 5 | 0  | 0   |
| Mild               | 0 | 0 | 3  | 0   |
| Moderate           | 0 | 1 | 4  | 4   |
| Severe             | 0 | 0 | 2  | 11  |

PCNA: Proliferating cell nuclear antigen.

Fig. 2. A) Histopathology of the ileum; desquamation at the lamina epithelialis (black arrows) and lymphocytolysis in Peyer’s patches (white arrows) are obvious; (H & E, scale bar = 500 μm). B) Higher magnification of the lesioned gut; desquamation at the lamina epithelialis (black arrow) and lymphocytolysis in Peyer’s patches (white arrows) can be observed; (H & E, scale bar = 200 μm). C) Parvovirus-positive immunoreaction in intestinal cells (arrows); (SBPM, scale bar = 100 μm). D) Proliferating cell nuclear antigen (PCNA) expression in crypt cells (arrows) in control and E) Infected dogs, (SBPM, scale bar = 100 μm). F) Caspase 3 expression in epithelial cells (arrows), (SBPM, scale bar = 50 μm).

Fig. 3. Distributions of the A) PCNA and B) Caspase 3 positive cell percentages between parvovirus infected and control groups.
Discussion

The CPV infection is a common and fatal disease that most commonly affects puppies. The infected dogs develop acute gastroenteritis and leukopenia. In this study, 26 of 30 cases were positive for CPV infection and the ages of the dogs ranged from 2 to 5 months. Our clinical findings were similar to those of previous studies and classical knowledge.

Parvoviruses may infect cells at any phase of the cell cycle; replication is dependent on cellular mechanisms that are functional only during nucleoprotein synthesis prior to mitosis. Hence, the effects of parvoviral infection are primarily manifested in tissues with a high mitotic rate. Parvovirus replication in dogs was primarily detected in lymphoid tissues and gastrointestinal tract epithelial cells. Therefore, the gut samples were used for this study. Histopathological findings of our study were in agreement with classical knowledge and edema, desquamation, erosion, ulcers and hemorrhages in guts as well as severe atrophy or total necrosis of the Peyer’s patches were frequently observed.

Recent studies have demonstrated that CPV induces apoptosis in cell cultures. However, the role of apoptosis in the pathogenesis of CPV infection in the intestine is unknown. Since caspase 3 is a key protease executing apoptosis, we investigated whether caspase 3 is expressed in the intestinal sections of dogs naturally infected with parvovirus. It was found that caspase 3 is indeed strongly expressed in some cells of the intestines. This result indicated that the apoptosis of intestinal cells appears to be mediated by caspase-dependent and in some cells independent pathways. In fact, an earlier study has suggested that other signaling pathways can induce apoptosis independent of the caspase cascade. These findings indicate that the necrosis in the intestines may be directly or adversely affected by caspase 3 activation.

Destruction of virus-infected cells through the induction of apoptosis is an important host defense mechanism that may serve to limit virus replication and spread within host tissues. We demonstrated here that parvovirus induces apoptosis in the intestinal cells primarily by inducing the caspase 3 pathway. We also found that both parvovirus and caspase 3 are expressed in crypt and epithelial cells of the villi. These results indicate a specific role for caspase 3 in parvoviral enteritis in dog intestinal cells.

In CPV infection, regeneration of the cryptic epithelium and partial or complete restoration of the mucosal architecture occur if undamaged stem cells persist in most of the affected crypt cells and the animal survives the acute phase of parvoviral enteritis. After the acute period, the infected dogs either succumb or begin to recover. The identification of PCNA as a processivity factor for replicative DNA polymerases has placed it at the heart of the replisome. However, an earlier study has revealed additional roles for this protein in coordinating the complex network of interactions at the replication fork. In this study, marked PCNA activity was observed in regenerated crypt and epithelial cells of the villi. Immunohistochemical measurement of the cells proliferative activity has been widely used to assess the biological behavior of tumors. The PCNA has been found to be useful for the diagnosis and evaluation of prognosis of patients suffering from a variety of malignant tumors. However, there is limited information regarding such measurements in viral infections. There have been no reports regarding PCNA in canine parvoviral enteritis till date. In the present study, increased PCNA activity was observed; while, it was not sufficient for complete healing. Therefore, the proportion of PCNA activity may be related to the survival and recovery of parvovirus-infected dogs.

In this study, increased expressions of PCNA and caspase 3 were observed in numerous intestinal cells in dogs with naturally induced parvoviral enteritis and this result indicated that both PCNA and caspase 3 have important roles and may be useful for the disease prognosis determination. Although PCNA expression was observed in regenerative intestinal cells, caspase 3 expression was observed in cells near the lesions. Since the positive reaction most commonly observed in crypt cells, it is believed that these cells have an important role in parvoviral enteritis. A major limitation of this study was the absence of hematological examination and laboratory results of the puppies.

In conclusion, the present study showed that CPV induces apoptosis in the intestinal cells and moreover, there were epithelia regenerations in dogs survived for a longer time. Our results suggest the presence of an intrinsic balance between apoptosis and cell proliferation in the intestinal cells of dogs with parvoviral enteritis.

Acknowledgments

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Conflict of interest

There is no conflict interest.

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