

Short communication

In vitro study of neutrophil apoptosis in dogs

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Abstract

In the present study, to investigate the apoptosis of the polymorphonuclear neutrophil (PMN) from healthy dogs, we carried out TUNEL assay and DNA analysis by electrophoresis on dog PMNs. The TUNEL assay indicated that apoptotic PMNs in dogs were $0.15 \pm 5\%$ before incubation, $0.3 \pm 5\%$ at 4 h incubation, $1 \pm 6\%$ at 8 h, $9 \pm 4\%$ at 12 h and $28 \pm 5\%$ at 24 h, respectively. The ladder formation was much more clearly observed in DNA from PMNs after 24 h incubation at 37°C than that before incubation. The results in this study indicated that healthy dog PMNs undergo apoptosis spontaneously within hours to days, and that the apoptosis of PMNs might be related to the high turnover of these circulating cells in dogs. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Neutrophil; Apoptosis; Dog; TUNEL assay; DNA ladder

1. Introduction

Apoptosis (programed cell death) is an active and well regulated process that is characterized by specific phenomena such as cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, membrane blebbing, and finally, the decay into apoptotic bodies (Kerr et al., 1972; Wyllie, 1980; Squier et al., 1995). The mature human polymorphonuclear neutrophil (PMN) undergoes apoptosis spontaneously within hours to days, and this is thought to contribute to the homeostasis of functional leukocyte pools and the high turnover of these cells in the circulation (Kerr et al., 1972; Payne et al., 1994; Weinmann et al., 1999). The life span of circulating PMNs is relatively short when compared with other leukocytes, and it can be further shortened or extended by accelerating or delaying apoptosis (Squier et al., 1995; Aoshiba et al., 1999). Upon induction of apoptosis, the plasma membrane of the PMNs remains intact, thus

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preventing the release of proinflammatory and histotoxic contents of these cells (Weinmann et al., 1999). Because apoptotic process also leads to an impairment of PMN responsiveness and allows specific recognition and elimination of the cells by phagocytosis, the induction or prevention of PMN apoptosis is currently discussed as a key event in the control of inflammation (Savill et al., 1989; Whyte et al., 1993; Hall et al., 1994; Weinmann et al., 1999).

However, the relationship of PMN apoptosis and inflammatory diseases in animals has not been well investigated. In the present study, to investigate the apoptosis of PMNs from healthy dogs, we carried out terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay and DNA analysis by electrophoresis on dog PMNs.

2. Materials and methods

2.1. PMNs isolation

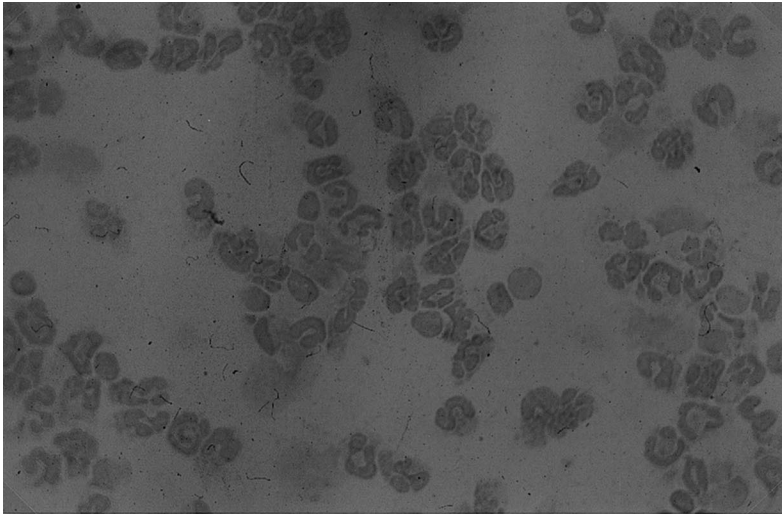
PMNs were isolated and cultured using the medium reported by Kusaba et al. (1998). Briefly, blood samples were collected from cervical vein of three healthy beagle dogs. 10 ml of EDTA treated blood samples supplemented with equal volume of dextran (dextran 200,000, 3% (w/v), Wako, Osaka, Japan) were allowed to stand for 30 min at room temperature to prepare leukocyte rich plasma following the sedimentation of erythrocytes. The leukocyte-rich plasma was centrifuged at 200 g for 10 min. The pellet, which contained granulocytes and erythrocytes were mixed for 40 s with 0.3% NaCl to destruct the erythrocytes, and then mixed with 1.5% NaCl. The resulting leukocyte-rich plasma was centrifuged at 200 g for 10 min. The pelleted cells were resuspended in 5 ml PBS and overlaid on 5 ml of Ficoll-Hypaque (Lymphoprep; specific gravity 1.077, NYCOMED PHARMA AS, Oslo, Norway), and then centrifuged at 400 g for 20 min. The pellets were suspended in RPMI 1640 medium added with 10% fetal calf serum. Final concentration of the cells were 4×10^6 cells/ml. The samples consisted of more than 95% PMNs and were incubated at 37°C for various time periods (0, 4, 8, 12 and 24 h).

2.2. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay

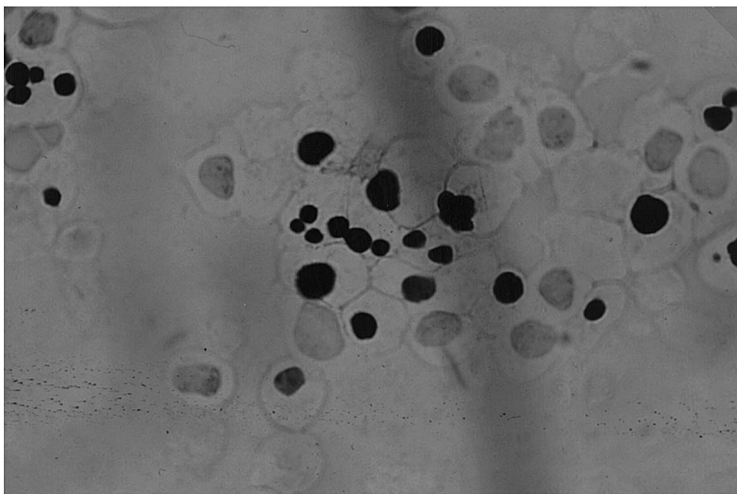
After incubation for various time periods (0, 4, 8, 12 and 24 h), neutrophils were washed twice with PBS and collected on poly-l-lysine-coated slides using cytospin and dried for 15 min. The samples were fixed with 10% neutral buffered formalin at room temperature for 25 min. The TUNEL assay was performed using DeadEnd Colorimetric Apoptosis Detection System, following the manufacturer's instructions (Promega Corporation, Madison, USA). For counterstain, 0.5% (w/v) methyl green in 0.1 M sodium acetate was used. Visualization of the bound horseradish-peroxidase-labeled streptavidin was detected using 3,3'-diaminobenzidine (DAB) (Gavrieli et al., 1992; Kusaba et al., 1998). At least 500 cells were evaluated in each preparation and the TUNEL-positive and -negative cells counted.

2.3. DNA electrophoresis

PMN cells (4×10^6) were collected from the flasks by scraping and centrifuged at 200 g for 10 min. The resulting pellets were resuspended in 400 μ l in a lysis buffer containing 5 mM Tris-HCl (pH 7.4), 0.5% SDS, 2 mM EDTA, 20 μ g of proteinase-K per ml and RNase (20 μ g), and incubated at 50°C for 1 h. High molecular weight DNAs were



A



B

Fig. 1. Morphological features of PMNs apoptosis during incubation. A: Neutrophils preincubation.($\times 400$). B: Neutrophils incubated for 24 h ($\times 400$). Visualization of the bound horseradish-peroxidase-labeled streptavidin bound to TUNEL-positive cells by 3,3'-Diaminobenzidine (DAB).

obtained from the PMNs sample by phenol and chloroform extraction. These DNA samples were dissolved in TE buffer (10 mM Tris-hydrochloride, pH 8.0 and 1 mM EDTA) and used for DNA electrophoresis. The extracted DNA samples (20 μg) were electrophoresed through 2% agarose gel and then stained with ethidium bromide (Ohno et al., 1994).

3. Results

3.1. TUNEL assay

Neutrophils exhibiting the morphological changes of apoptosis are demonstrated in Fig. 1. Morphological features of apoptosis in neutrophils immediately after isolation from dogs were hardly present (Fig. 1A). After 24 h, neutrophils had round nuclei with no lobules (Fig. 1B). The incidence of apoptotic cells in dog PMNs increased with time of incubation (Fig. 2).

3.2. Electrophoretic analysis of DNA

Fig. 3 shows representative results of gel electrophoresis of DNA from incubated PMNs. After 24 h incubation, a ladder formation of DNA fragments of 180–200 bp was

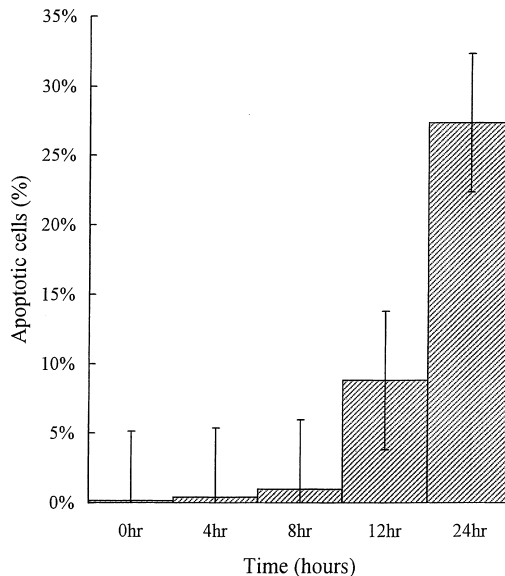


Fig. 2. Time course for apoptosis of incubated PMNs in three dogs. The results are expressed as mean \pm S.D. Apoptosis was significantly increased for neutrophils from 24 h incubation compared to preincubation. The incidence of apoptotic cells in dog PMNs were 0.15 \pm 5% before incubation, 0.3 \pm 5% at 4 h incubation, 1 \pm 6% at 8 h, 9 \pm 4% at 12 h and 28 \pm 5% at 24 h, respectively.

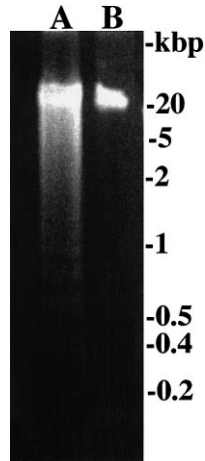


Fig. 3. Gel electrophoresis of DNA from incubated PMNs. Lane A: The characteristic DNA ladder formation appeared at 24 h incubation. Lane B: DNA from neutrophils appeared at preincubation.

clearly exhibited (Fig. 3). This ladder formation of the DNA from dog PMNs was gradually more clear depending on the incubation time.

4. Discussion

The results in this study indicated that healthy dog PMNs like human PMNs undergo apoptosis spontaneously within hours to days, suggesting the high turnover of these cells in the circulation. These apoptosis cells were determined by the criteria of Savill et al. (1989) by light microscopy, however, no necrotic cells were detected in this study. It is necessary to control the inflammation, avoiding unwanted tissue damage caused by activated neutrophils (Kerr et al., 1972). Kusaba et al. reported that the life span of the PMNs in human cirrhotic patients was shortened by accelerated apoptosis *in vitro* (Kusaba et al., 1998). They suggested that these findings might account for the neutropenia observed in cases with cirrhosis and explain the increased incidence rate of bacterial infections seen in this disease (Kusaba et al., 1998). However, it has not been determined that the life span of PMNs of dogs with inflammatory or non-inflammatory diseases is shortened by accelerated apoptosis. A comparison of the incidence of PMNs apoptosis in patient dogs is now under examination in our laboratory.

The molecular mechanism that controls PMNs apoptosis remains widely unknown. Recent studies on neutrophil apoptosis indicated the involvement of tryosine phosphorylation events in signaling pathways. Some cytokines affect the ratio of Bax- α /Bcl-X1 expression in human PMNs and modulate the subsequent activity of caspase-3, which functions as an executor of programmed cell death and may promote apoptosis (Weinmann et al., 1999).

Further investigations on the relationship of PMNs apoptosis and clinical diseases in animals are required.

References

- Aoshiba, K., Yasui, S., Hayashi, M., Tamaoki, J., Nagai, A., 1999. Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils. *J. Immunol.* 162, 1692–1700.
- Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119, 493–501.
- Hall, S.E., Savill, J.S., Henson, P.M., Haslett, C., 1994. Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin. *J. Immunol.* 153, 3218–3227.
- Kerr, J.F.R., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26, 239–257.
- Kusaba, N., Kumashiro, R., Ogata, H., Sata, M., Tanikawa, K., 1998. In vitro study of neutrophil apoptosis in liver cirrhosis. *Intern. Med.* 37, 11–17.
- Ohno, K., Okamoto, Y., Miyazawa, T., Mikami, T., Watari, T., Goitsuka, R., Tsujimoto, H., Hasegawa, A., 1994. Induction of apoptosis in a T lymphoblastoid cell line infected with feline immunodeficiency virus. *Arch. Virol.* 135, 153–158.
- Payne, C.M., Glasser, L., Tischler, M.E., Wyckoff, D., Cromey, D., Fiederlein, R., Bohnert, O., 1994. Programmed cell death of the normal human neutrophil: an in vitro model of senescence. *Microsc. Res. Tech.* 28, 327–344.
- Savill, J., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M., Haslett, C., 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.* 83, 865–875.
- Squier, M.K.T., Sehnert, A.J., Cohen, J.J., 1995. Apoptosis in leukocytes. *J. Leukocyte Biol.* 57, 2–10.
- Weinmann, P., Gaehtgens, P., Walzog, B., 1999. Bcl-Xl-and Bax- α -mediated regulation of apoptosis of human neutrophils via caspase-3. *Blood* 93, 3106–3115.
- Whyte, M.K.B., Meagher, L.C., MacDermot, J., Haslett, C., 1993. Impairment of function in aging neutrophils is associated with apoptosis. *J. Immunol.* 150, 5124–5134.
- Wyllie, A.H., 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284, 555–556.