Apoptosis of neutrophilic granulocytes of bovine virgin mammary gland in scanning electron microscopy

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ABSTRACT: The objective of this work was the morphologic analysis of apoptosis of neutrophilic granulocytes (hereinafter referred to as neutrophils) in scanning electron microscopy (SEM) in comparison with morphological features distinguishable by light microscopy. This study was performed on 12 bovine virgin mammary glands washed with physiological buffered solution (PBS) prior to the induction of cell influx by PBS. Twenty-four hours after influx induction the cell suspension was obtained by the lavage of mammary glands with PBS. The particular lavages were cytologicaly and bacteriologicaly examined – all bacteriological examinations were negative. Mononuclear phagocytes (MoP), lymphocytes and neutrophils were distinguished in the cell suspension of the lavages by means of light microscopy. The neutrophils predominated in differential cell count. Neutrophil population showed some signs of structural features typical for the process of apoptosis that were distinguished in haemocytometer and light microscopy on stained microscopical smears. The process of apoptosis consisted of three structurally different stages: karyopyknosis, zeiosis and the stage of apoptotic bodies. These stages of neutrophil apoptosis were distinguished also by SEM. Karyopyknotic neutrophils assumed spherical shape while they lost all of their superficial pseudopodia. Neutrophils in zeiosis stage showed prominent surface protumberances, bubble-shaped vesicles causing a bizarre deformation of the cells. After the membrane vesicles had split off, they began to form spherical formations (apoptotic bodies). On the basis of neutrophils' specific structural properties it could easily distinguish all the three stages of neutrophil apoptosis by means of SEM technique as well as other morphological methods.

Keywords: apoptosis; neutrophilic granulocyte; scanning electron microscopy; bovine virgin mammary gland

INTRODUCTION

Neutrophilic granulocytes (hereinafter referred to as neutrophils) play an important role in phagocytic defensive system of bovine mammary gland. These cells circulate in blood and then they leave the blood stream under the pressure of chemotactic substances. Finally they migrate through the tissues to mammary gland where they perform their phagocytic function (Paape and Wergin, 1977).

The life cycle of neutrophils in body tissues is relatively short; neutrophils are predisposed to apoptosis, which destroys them within 1 to 2 days after they leave the bloodstream (Raff, 1992). Formerly, the necrosis was considered to be the only way of neutrophil destruction in bovine mammary gland (Nickerson *et al*., 1986; Paape *et al*., 1991).

However, we noted that neutrophils are not death only by necrosis in the cavitary system of virgin mammary gland but also, and even in a greater extent, by apoptosis (Sládek and Ryšánek, 1999a, 2000). In the works mentioned the neutrophils were studied above all in light, fluorescence, confocal and transmission electron microscopy. Surprisingly, the method of scanning electron microscopy has never been used for studying apoptosis of neutrophils in bovine mammary gland although the superficial structure of neutrophils may indicate their function (Nickerson *et al*., 1986; Paape *et al*., 1991).

Therefore we decided to perform a morphological analysis of neutrophil apoptosis of bovine mammary gland in scanning electron microscopy (SEM) in comparison with morphological features distinguishable by light microscopy.

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MATERIAL AND METHODS

Animals and experimental design

The study was carried out in 12 clinically normal virgin Holstein × Bohemian Red Pied heifers aged 16 to 18 months. The heifers were kept in an experimental littered stanchion barn and fed a standard diet consisting of hay and mineral-supplemented concentrates. The model of induced influx was used in order to increase the yield and purity of neutrophils (Wardley *et al*., 1976). Obtained cell suspensions were put to bacteriological and cytological examinations and the total differential counts of somatic cells were determined. Afterwards, neutrophil population was studied in light and scanning electron microscopy.

The model of induced influx

All mammary glands of the heifers were washed with 20 ml of PBS, pH 7.4, warmed up to 37°C. The washing was followed by intracisternal administration of 10 ml of PBS. Twenty-four hours later the cells were collected by means of another lavage with 20 ml of PBS warmed up to 37°C.

Cells processing

Bacteriological examination of all the lavages, by culture on blood agar plates (5% washed sheep erythrocytes) and aerobic incubation at 37°C for 24 h, yielded invariably negative results. Total mammary cell counts were determined using a haemocytometer. The trypan blue dye exclusion test demonstrated 98% cell viability. The cell suspensions were centrifuged at 4°C and 200 × g for 10 min. One millilitre of supernatant was retained for resuspension of the pellet the remaining supernatant was recanted.

Light microscopy

Smears of cell suspensions for light microscopy were prepared by the standard haematological procedure, dried and stained with Pappenheim (Bessis, 1973). Differential cell counts were determined by counting at least 200 cells in each smear. Relative representation of apoptotic neutrophils was determined by the above-described method (Sládek and Ryšánek, 2000).

Scanning electron microscopy

The cells for SEM were processed by method of Paape and Wergin (1977). Briefly, fresh cell suspensions were added on cover slips and incubated for 30 min at 37°C in thermostat. Then the cells were fixed with 3% glutaraldehyde in cacodylate buffer (0.1M) for 15 min on cover slips. The wet preparations were rinsed with distilled water, dehydrated in a graded series of ethanols resolved in amyl acetate and dried by the carbon dioxide critical point procedure and examined in the SEM Tesla BS 300 (Tesla a.s., Brno, Czech Republic). Electronograms were digitalised and analysed by Lucia M software (Laboratory Imaging, Prague, Czech Republic).

RESULTS

Virgin mammary gland cytology

The lavages obtained from mammary glands after induced influx of the cells were coloured grey-cloudy owing to a great number of the cells present ($56.9 \pm 12.8 \times 10^6$ per ml standardised to the volume of 20 ml PBS). Light microscopy distinguished three types of leucocytes in the cell population – a great deal of neutrophils (87.9 ± 8.1%) and a lesser amount of mononuclear phagocytes (8.7 ± 2.4%) and lymphocytes (3.4 ± 2.3%). Within the neutrophil population, a detailed morphological haemocytometer and light microscopy analysis identified the cells with apparent structural features typical for apoptosis.

Apoptosis of neutrophil in light microscopy

In light microscopy on stained smears, structurally normal neutrophils appeared to form middle-sized cells (7 to 10 μm) with lobe-shaped nuclei, neutral granules in pink cytoplasm and pseudopodia on the surface (data not shown, for details see in Sládek and Ryšánek, 1999a). Next to them apoptotic neutrophils were distinguished. The apoptotic process of neutrophils was characterised by three structurally different stages: karyopyknosis, zeiosis and the fragmentation into apoptotic bodies. In karyopyknosis stage, there formed the small spherical apoptotic cells with smooth surface and spherical nuclei containing densely condensed chromatin (Figure 1). The cells with protuberances on the surface (i.e. superficial membraned bizarrely shaped vesicles) were typical for zeiosis stage (Figure 1). Finally, apoptotic neutrophils in the form of small apoptotic bodies about 3 μm long were distinguished (Figure 1).

Apoptotic neutrophils on stained smears and, even sooner, on haemocytometer examination of the cell population were observed. Stage of zeiosis of apoptotic neutrophils was apparent in haemocytometer often (Figure 2). All three stages of apoptosis of bovine virgin mammary gland neutrophils (their ratio is stated in Table 1) were then subjected to SEM study.

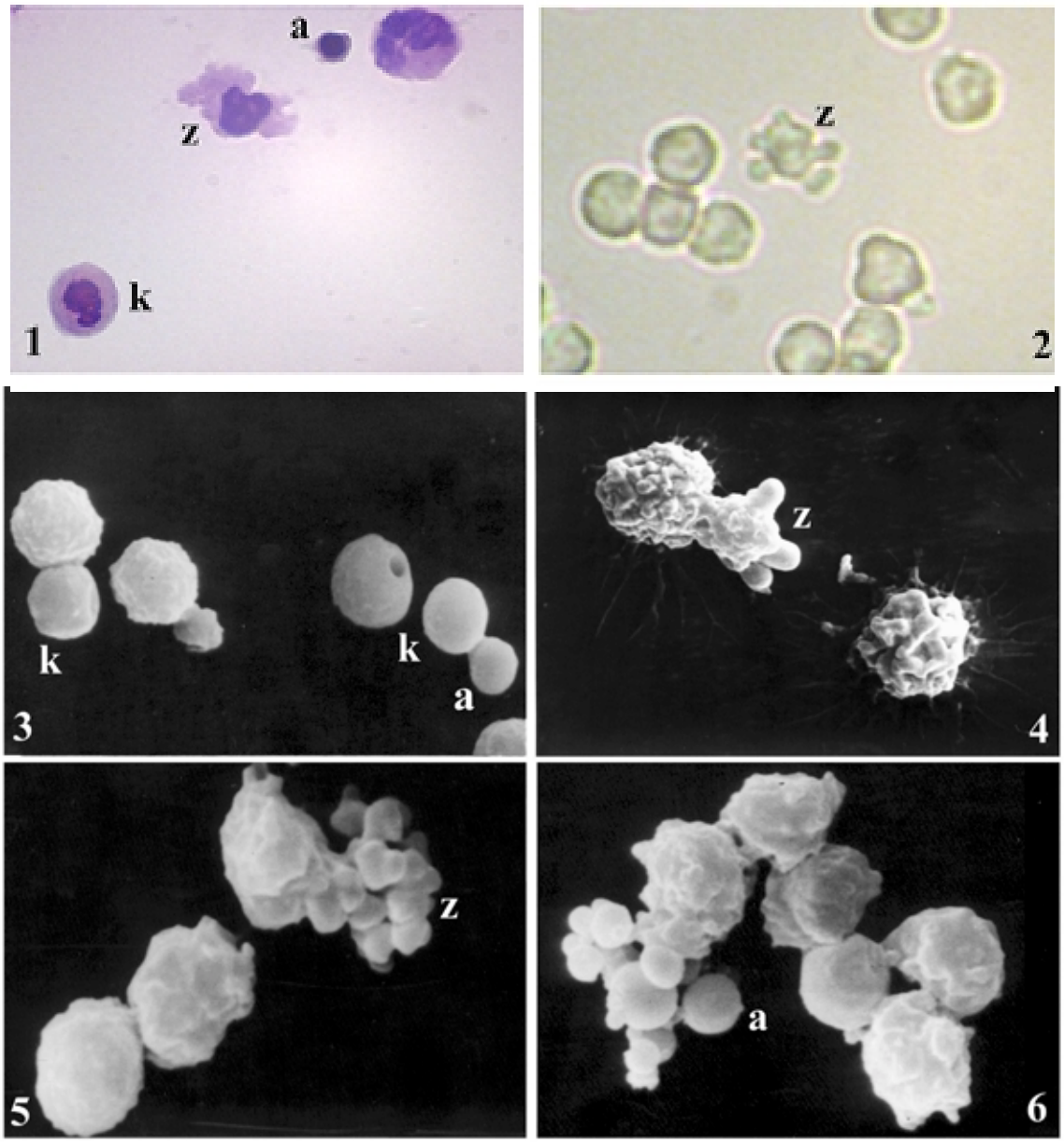

Figure 1–6. Light microscopic and scanning electron microscopic pattern of apoptosis of neutrophils isolated from bovine virgin mammary gland. Karyopyknosis (k), zeiosis (z) and apoptotic body (a) in light microscopy on stained microscopic smear (Figure 1) and zeiosis (z) in haemocytometer (Figure 2). The equivalent stages of neutrophil apoptosis were represent in scanning electron microscopy: karyopyknosis (k, Figure 3), zeiosis (z, Figure 4 and 5) and fragmentation into apoptotic bodies (a, Figure 3 and 6). Original magnification: 1 000 × (Figure 1 and 2), 2 500 × (Figure 3 and 4), 4 000 × (Figure 5 and 6)

Apoptosis of neutrophil in scanning electron microscopy

The neutrophils with normal ultrastructure predominated in SEM as well as in light microscopy on stained smears. These cells either formed clusters or they were solitary. Their ultrastructure was characterised by spherical or slightly ovoid, oblate or semi-ovoid shape in dependence on the degree of adherence. The non-adhesive cells had mostly a spherical shape while the adhesive cells were rather hemispheric or semi-ovoid. The surface of all cells was formed by short conic pseudopodia while the adhesive neutrophils formed also the stretched filaments at their basis that resembled long radial fibrous filamentes (Figure 4).

Similarly to light microscopy analysis, the process of neutrophil apoptosis was characterised by three structurally different stages in SEM too.

The early neutrophil apoptosis stage in SEM (karyopyknosis in light microscopy, see above) was characterised

Table 1. Proportion of karyopyknosis, zeiosis and apoptotic bodies in neutrophil population from bovine virgin mammary glands after induced influx

Karyopyknotic neutrophils (%)	6.6 ± 2.8
Zeiosis (%)	0.9 ± 0.6
Apoptotic bodies (%)	0.5 ± 0.1

by size and shape changes of the cells and the changes of cell surface design. The cells were reducing in size while assuming a spherical shape. However, a total loss of pseudopodia including the stretched filaments was observed (Figure 3).

In the consequential apoptosis stage – zeiosis – the spherical cells formed prominent membraned, bubble-shaped vesicles causing a bizarre deformation of the cells (Figure 4). The number of these vesicles was directly proportional to time; i.e. their number was increasing gradually. Later the vesicles got strangulated at their bases and the shape of apoptotic neutrophils became grape-like in late zeiosis stage (Figure 5). The zeiosis of apoptotic cells finally transformed to the terminal apoptosis stage, i.e. the stage of apoptotic bodies.

As the membraned vesicles (mutually linked by thin bridges at the beginning) got gradually strangulated and detached, they changed into the apoptotic bodies about 3 μm long. Finally, the solitaire apoptotic bodies mingled with other cells (Figures 3 and 6).

DISCUSSION

The objective of this work was the morphologic analysis of apoptosis of neutrophils in scanning electron microscopy (SEM) in comparison with morphological features distinguishable by light microscope. Thus it completes our previous work focusing on primary morphological description of heifers' mammary gland neutrophils in light, fluorescence, confocal and transmission electron microscopy (Sládek and Ryšánek, 2000).

Neutrophils play an important role in defensive system of bovine mammary gland, their function is phagocytosis of pathogens. They are armed with the enzymes residing in azurophile and specific granules of their cytoplasm. If the enzymatic content of the granules is released from the cell, it destroys also the surrounding tissues (Hurley, 1983). This happens especially on the necrosis of neutrophils. Today it is generally accepted that apoptosis is induced in order to prevent the outflow of histotoxic content of neutrophils granules. Unlike necrosis, apoptosis does not cause the outflow of granule content to extracellular space (Savill, 1997; Whyte *et al*., 1999). Moreover, it has been proved that neutrophils are predisposed especially to apoptosis after they leave the bloodstream (Raff, 1992).

The process of apoptosis has been described morphologically for most of the types of cells (Wyllie *et al*., 1980) and it has been observed that it consist of the following three consequential stages: karyopyknosis, zeiosis and the fragmentation to apoptotic bodies promptly phagocytised by local macrophages. The stages mentioned above were described for the first time in human arthritis exudate (Savill *et al*., 1989). Later it was observed in human clinical material and experimental material of laboratory animals (for review see Sládek and Ryšánek, 1999c).

Conversely, the past studies on neutrophils of lactating mammary gland noted only degenerated and necrotic cells in addition to structurally normal neutrophils (Nickerson *et al*., 1986; Lintner and Eberhart, 1990; Paape *et al*., 1991). Later on, the processes of neutrophil degeneration and necrosis were seen also in bovine virgin mammary gland (Sládek and Ryšánek, 1999a, b). Moreover, in neutrophil population of bovine virgin mammary gland we observed also neutrophils that showed structural and ultrastructural changes typical for apoptosis (Sládek and Ryšánek, 2000). This fact confirmed our assumption that neutrophils of cavitary system of bovine mammary gland die due to both necrosis and mainly apoptosis.

In above-mentioned work we found out that the process of neutrophil apoptosis starts with the reduction of cell's size and the loss of pseudopodia. Nucleus desegmentation and chromatin condensation (karyopyknosis) comes simultaneously and as early as in this stage the phagocytosis of apoptotic neutrophils by MoP is started; or the cells are changed morphologically into bizarre forms with numerous superficial protuberances, i.e. vesicles (zeiosis). As we have already mentioned, the elementary examination of the cell population of bovine virgin mammary gland in haemocytometer showed apparent structural features resembling above all the stage of zeiosis. Similar data were published in a work studying the interaction of neutrophils in bovine blood with bacteria *Haemophilus somnus* (Yang *et al*., 1998). This fact forced us to analyse the structural abnormalities by SEM that allowed us to study ultrastructural properties of cell's surface. Another reason for our quest was also the absence of any literature providing SEM ultrastructural analysis of apoptosis of bovine mammary gland neutrophils.

Three stages of apoptosis were observed in SEM. The stage titled in light microscopy as karyopyknosis was in SEM characterised by spherical shape of the cells and a total loss of pseudopodia. The cells had completely smooth surface and they reduced in size. In next apoptosis stage, these karyopyknotic neutrophils formed superficial protuberances (membraned vesicles) progressively while they assumed rather bizarre shapes. The membraned vesicles strangulated gradually but remained interconnected by thin bridges. In SEM, these

formations resembled the grapes of wine. In light and transmission electron microscopy, these membraned vesicles showed apparent fragments of nucleus with densely condensed chromatin (Sládek and Ryšánek, 2000). Gradual strangulation disconnected the thin bridges while the vesicles became free and started to form the apoptotic bodies. Apoptotic neutrophils, together with apoptotic bodies, were finally phagocytised by MoP (Savill *et al*., 1989; Sládek and Ryšánek, 2000).

It is obvious then that SEM can clearly differentiate all the three stages of neutrophil apoptosis of bovine mammary gland – as well as the methods detecting apoptosis on the basis of staining and contrasting the cells. It is due to the fact that apoptosis causes the change of cell's size and shape mainly.

Apoptosis of cells is a very dynamic process that lasts only several hours in dependence on cell type (Majno and Joris, 1995). Although the aim of this work was not to specify the dynamism of neutrophil apoptosis, from the particular ratios we might deduce the following conclusion. Most of apoptotic cells in neutrophil population of bovine virgin mammary gland are represented by karyopyknotic cells, then come zeiotic cells while the apoptotic bodies are present in the smallest amount. Therefore the stage of karyopyknosis would be probably relatively longer in comparison to the stage of zeiosis and the stage of apoptotic bodies. However, this hypothesis should be confirmed by a deeper investigation of the dynamism of neutrophil apoptosis, especially under exactly defined conditions *in vitro*.

In conclusion we may assert that there are the cells in neutrophil population in bovine virgin mammary gland that show ultrastructure properties typical for the process of apoptosis. SEM, as well as other morphological techniques, clearly distinguishes the three stages of neutrophils apoptosis according to neutrophils' specific surface properties.

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