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Apoptosis in ovarian granulosa cells of cattle: Morphological features and clearance by homologous phagocytosis

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ABSTRACT

Apoptosis is involved in many physiological processes of the ovary, such as recruitment of prenatal germ cells, follicular atresia, ovulation, and luteolysis. Based on the need for the involvement of phagocytic cells to achieve apoptosis clearance and that follicular atresia is triggered by weak apoptotic stimuli, we postulate that granulosa cells engullng apoptotic corpses (ACs) must carry out this macrophagic process. Since apoptosis was early defined in terms of morphological aspects, here we describe apoptosis induced by a GnRH analog (leuprolide acetate, LA) at histological level on bovine granulosa cells (primary culture, CPGB, and an established cell line, BGC-1). We observed two main types of apoptosis. In type A, the whole cell or most of it is compacted into a single large AC that is then engulfed by neighboring cells or simply detached. In type B, small portions of cells, either with or without nuclear material, become ACs that are also phagocytosed. Apoptosis and homologous phagocytosis were confirmed by TUNEL and immunocytochemistry for Bax and active caspase 3. Induction of apoptosis was significant in BGC-1 cells treated for 24 h with 100 nM LA. CPGB cells showed two types of response with different doses of LA. Fetal calf serum was necessary to find apoptosis induced by LA.

Introduction

Cell death is an integral cellular process that mainly occurs by two major mechanisms. Apoptosis is a programmed, energy-dependent death process that causes typical morphological changes including cell shrinkage, nuclear condensation, DNA fragmentation and membrane alterations. Apoptosis usually affects scattered cells in a tissue and is triggered by stimuli in both, physiological and pathological settings. When cells die by apoptosis they are either released in the tissue environment or more commonly, engulfed by neighboring cells or by specialized phagocytes, including macrophages, dendritic cells or neutrophils.

Clearance of apoptotic cells, also termed efferocytosis, is mediated by professional and amateur phagocytes (Ablin et al., 2005). Although these cells are believed to remove most of the apoptotic corpses (ACs), it is becoming increasingly evident that essentially any cells can take up neighboring dying cells (Williams et al., 2005). However, amateur phagocytes or less mobile 'resident' cell types typically engulf ACs with much slower kinetics than professional phagocytes (Clark et al., 2002). Although a substantial amount of research has produced significant accomplishments in identifying molecules involved in the engulfment of apoptotic cells, the exact role and timing of individual molecules during this process remains unclear (Steinman et al., 2000; Duffield, 2003; Sun et al., 2004). Numerous *in vitro* assays have been done to elucidate the mechanism by which macrophages clear ACs, and various membrane cell markers have been identified (Fadok et al., 2000; Li et al., 2003). We were interested in understanding the non-professional clearance of apoptotic cells by neighboring cells *in vitro* by homologous cell phagocytosis and we used ovarian follicular granulosa cells (GC) as a suitable model for these aims.

Apoptosis is involved in many physiological processes of the ovary, such as recruitment of prenatal germ cell (oocyte death), follicular atresia (death of GC), ovulation (death of follicular epithelial cells), and luteolysis (luteal cell death) (Tilly et al., 1991; Hussein, 2005). The small size of AC, their short half-life and phagocytic clearance contribute to claims that apoptosis is a rare event in

Abbreviations: AC, apoptotic corps; CL, corpus luteum; CPGB, bovine granulosa primary culture; GC, granulosa cells; GnRH, gonadotropin releasing hormone; ICC, immunocytochemistry; LA, leuprolide acetate; ORO, oil red O.

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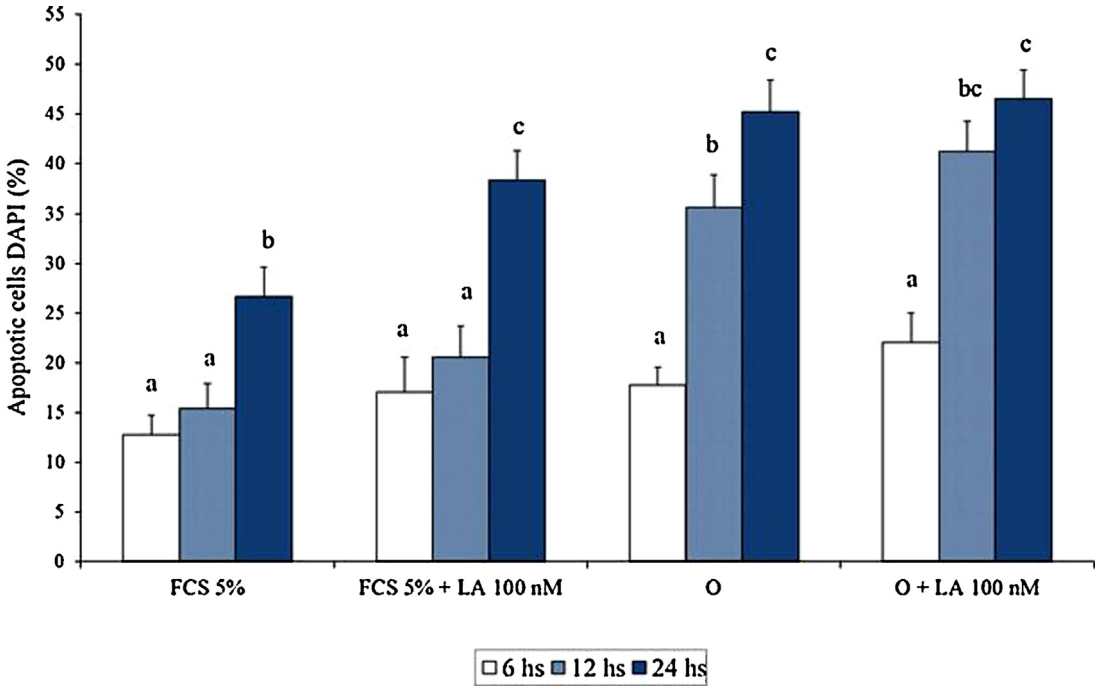

Fig. 1. Apoptosis measured by staining with DAPI in BGC-1 cells treated with or without leuprolide acetate (LA) 100 nM in the presence (5% FCS) or absence (0) 5% FCS (fetal calf serum) with 6, 12 and 24 h of incubation. Three experiments by triplicate ($n = 9$) were performed. The effect of time of incubation in the presence or absence of 5% FBS was analyzed, and the effect of LA for each incubation time in the presence or absence of 5% FCS. Increase in the percentage of apoptotic figures due to treatment with LA was observed in the presence of FBS 5% with 24 h of incubation ($p = 0.0001$). Spontaneous apoptosis was observed at 12 h ($p < 0.0001$) and 24 h ($p = 0.0001$) of incubation.

histological observations by light microscopy. In contrast, in cell cultures it is more frequently seen. AC beyond the phagocytosis in culture, lose their integrity after 1 h, resulting in swelling, loss of density, and membrane rupture freeing organelles that cause the so-called secondary necrosis (late apoptosis), which is distinguished from vital dye-excluding early apoptotic cells (Cummings et al., 1997). Owing to the lack of phagocytic counterparts, Majno and Joris (1995) and Gregory and Pound (2010) observed secondary necrosis associated with apoptosis in several cell culture lines. The absence of removal of AC that is observed *in vitro* has led to the development of techniques using microparticles to stimulate the purification of living cells during culture, or to engage the preincubation of the cell lines with macrophages to enhance their survival (Gregory and Pound, 2010). These authors have not included GC in their study and we believe that using GC in the presence of FCS could prevent this drawback. Phagocytosis of AC carried out by GC *in vivo* has been described in sections of ovaries by electron microscopy (Cummings et al., 1997). In animal and human ovaries, macrophages have been found in thecal, luteal and interstitial tissues of late atretic follicles (Best et al., 1996; Hume et al., 1984; Petrovska et al., 1996; Gaytan et al., 1998; Takaya et al., 1997; Li et al., 1998). In the mouse, macrophages were more abundant in the corpus luteum (CL) and atretic follicles, and their number fluctuated during the estrus cycle with highest amounts in proestrus and metaestrus, indicating that phagocytosis in the ovary is associated with hormonal regulation (Brannstrom et al., 1993; Petrovska et al., 1996). The location and specific dynamics of macrophages and the wide diversity of their functions have been taken as indicative of their participation in several processes occurring in the ovary (Wu et al., 2004). However, the role of macrophages in ovarian functions *in vivo* seems to be controversial. Macrophages have not been found between the GC of healthy follicles, and there is no evidence of AC phagocytosis conducted by macrophages in early atretic follicles, so that GC may be just responsible for efferocytosis in early atretic follicles (Bonilla-Musoles et al., 1975).

Based on the need for the involvement of phagocytic cells in physiological apoptotic process to culminate in cell death (Lang and Bishop, 1993; Little and Flores, 1993; Diez-Roux and Lang, 1997; Geske et al., 2001), and that the process of follicular atresia in the ovary is triggered by weak apoptotic stimuli (such as low hormone levels or decrease in growth factors), we propose a model for studying physiological processes of the ovary that involve apoptosis.

We describe in detail the morphological aspects of the apoptosis induced *in vitro* in bovine granulosa cells by a GnRH analog (leuprolide acetate) by staining with DAPI, hematoxylin, TUNEL and immunocytochemistry (ICC) and cytochemical analysis determining phosphatidyl serine exposure. We present, for the first time, the clearance of apoptotic cells *in vitro* by amateur cells, namely granulosa cells from bovine ovary CPGB (primary culture), and BGC-1 (established granulosa cell line from bovine follicles). We purpose GC as a suitable model for the future analysis of mechanisms for non-professional AC clearance *in vitro*.

Materials and methods

Cell culture

We worked in the proliferative area near the "plateau" of the cell growth curve in both cell types (BGC-1 and CPGB), in order to favor the induction of apoptosis against mitotic counterpart.

Established bovine granulosa cell line (BGC-1)

BGC-1 cells were used at a density of 50,000 cells/mL (maintaining 310 cells/mm^2) and a growth time of 24 h prior to induction. To evaluate morphology by staining with DAPI, hematoxylin and TUNEL techniques, and immunocytochemistry (ICC) for Bax and caspase 3, cells were seeded on 18 × 18 mm coverslips in multiwell plates. The culture media and conditions were DMEM + F12 (Sigma) supplemented with 2.4 g of sodium bicarbonate (pH 7.4), 5% FCS (fetal calf serum of biotechnological quality, Internegocios), 2 nM L-glutamine (Sigma), 50 mg/L gentamicin (Sigma), 5% CO_2 humidified

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