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Article title: Human decidual stromal cells secrete soluble pro-apoptotic factors during decidualization in a cAMP-dependent manner

First Author: E. Leno-Durán

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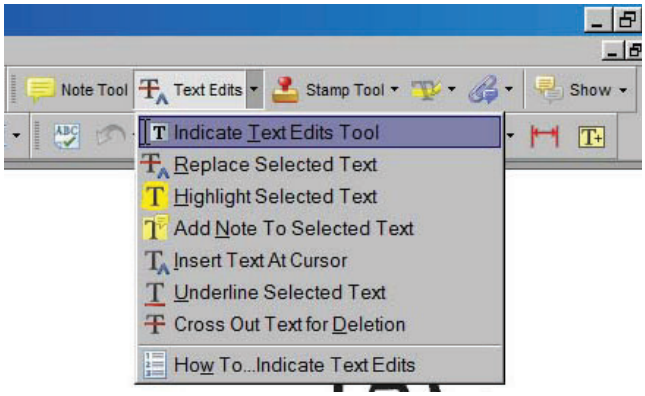

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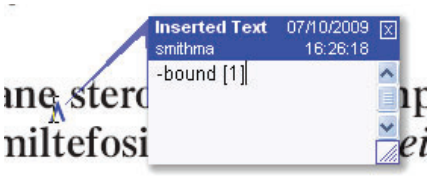

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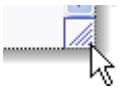

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human reproduction

ORIGINAL ARTICLE ***Reproductive biology***

Human decidual stromal cells secrete soluble pro-apoptotic factors during decidualization in a cAMP-dependent manner

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STUDY QUESTION: Is there a relationship between decidualization and apoptosis of decidual stromal cells (DSCs)?

SUMMARY ANSWER: Decidualization triggers the secretion of soluble factors that induce apoptosis in DSC.

WHAT IS KNOWN ALREADY: The differentiation and apoptosis of DSC during decidualization of the receptive decidua are crucial processes for the controlled invasion of trophoblast in normal pregnancy. Most DSC regress in a time-dependent manner, and their removal is important to provide space for the embryo to grow. However, the mechanism that controls DSC death is poorly understood.

STUDY DESIGN, SIZE, DURATION: The apoptotic response of DSC was analyzed after exposure to different exogenous agents and during decidualization. The apoptotic potential of decidualized DSC supernatants and prolactin (PRL) was also evaluated.

PARTICIPANTS/MATERIALS, SETTING, METHODS: DSC lines were established from samples of decidua from first trimester pregnancies. Apoptosis was assayed by flow cytometry. PRL production, as a marker of decidualization, was determined by enzyme-linked immunosorbent assay.

MAIN RESULTS AND THE ROLE OF CHANCE: DSCs were resistant to a variety of apoptosis-inducing substances. Nevertheless, DSC underwent apoptosis during decidualization in culture, with cAMP being essential for both apoptosis and differentiation. In addition, culture supernatants from decidualized DSC induced apoptosis in undifferentiated DSC, although paradoxically these supernatants decreased the spontaneous apoptosis of decidual lymphocytes. Exogenously added PRL did not induce apoptosis in DSC and an antibody that neutralized the PRL receptor did not decrease the apoptosis induced by supernatants.

LIMITATIONS, REASONS FOR CAUTIONS: Further studies are needed to examine the involvement of other soluble factors secreted by decidualized DSC in the induction of apoptosis.

WIDER IMPLICATIOSN OF THE FINDINGS: The present results indicate that apoptosis of DSC occurs in parallel to differentiation, in response to decidualization signals, with soluble factors secreted by decidualized DSC being responsible for triggering cell death. These studies are relevant in the understanding of how the regression of decidua, a crucial process for successful pregnancy, takes place.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Consejería de Economía, Innovación y Ciencia, Junta de Andalucía (Grant CTS-6183, Proyectos de Investigación de Excelencia 2010 to C.R.-R.) and the Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Spain (Grants PS09/00339 and PI12/01085 to E.G.O.). E.L.-D. was supported by fellowships from the Ministerio de Educación y Ciencia, Spain and the University of Granada. The authors have no conflict of interest.

Key words: DSC / apoptosis / decidualization

Introduction

The human decidua is the maternal component of the placenta that is in close contact with the fetal trophoblast. It is a heterogeneous tissue, which comprises decidual stromal cells (DSCs) as well as luminal and glandular epithelial cells and leukocytes. Several functions have been attributed to the decidua, such as an immunomodulatory role and the ability to regulate proliferation, differentiation and invasion of the trophoblast during pregnancy (Bulmer *et al.*, 1988; Dimitriadis *et al.*, 2010).

The decidualization of DSC is a process of differentiation and growth essential for successful implantation. This process initiates during the secretory phase of the menstrual cycle and continues if pregnancy occurs (Gellersen *et al.*, 2007). Undifferentiated DSCs (or preDSC), which are present in both the endometrium and decidua (Richards *et al.*, 1995; Olivares *et al.*, 1997), undergo morphological and phenotypic changes during decidual differentiation. They enlarge steadily and change their morphology from fibroblast-like to round. In addition, decidualized DSCs secrete factors such as prolactin (PRL), insulin-like growth factor-binding protein-1 and cytokines such as IL-15 and IL-11, which mediate the immunological functions of these cells (Dunn *et al.*, 2003). The decidualization process is induced by progesterone (P4) together with a group of local and endocrine factors, including prostaglandin estradiol (E_2), relaxin and gonadotrophins, which increase cellular levels of cAMP (Brosens *et al.*, 1999; Gellersen and Brosens, 2003). The role of cAMP in decidualization has been established in studies of DSC differentiation in culture (Telgmann *et al.*, 1997; Gellersen and Brosens, 2003).

Embryonic growth is accompanied by remodeling and involution of the decidua. The usual physiological mechanism to eliminate unnecessary, dangerous or redundant cells during development and tissue remodeling is apoptosis, a genetically controlled cell death process (Kerr *et al.*, 1972). In keeping with this, several studies have shown that regression of the decidua occurs by apoptosis (Gu *et al.*, 1994; Kayisli *et al.*, 2003; Mikhailov, 2003; Chan *et al.*, 2007). Cytokines and factors within the maternal–fetal environment may be involved in this process. In particular, death ligands CD95L and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expressed on fetal trophoblast cells have been proposed to induce apoptosis in cells of the materno-fetal interface upon binding to their respective death receptors (Uckan *et al.*, 1997; Chen *et al.*, 2004). However, the precise mechanism by which decidual cells undergo apoptotic cell death during pregnancy is not completely understood. Moreover, the relationship, if any, between decidualization and DSC apoptosis has not yet been investigated.

In this study, we analyzed the induction of apoptosis in decidualized in culture by treatment with P4, 8-bromo-cAMP and E_2. We provide evidence that in addition to its involvement in decidualization, cAMP plays a major role in the apoptotic cell death of decidualized DSC. Upon decidualization, DSCs secrete soluble factors capable of inducing apoptosis in differentiated and non-differentiated cells. Moreover, decidualized DSCs produce factors that increase lymphocyte survival. From a clinical point of view, understanding how apoptosis occurs in DSC during decidualization is important to advance our knowledge of pregnancy disorders.

Material and Methods

DSC isolation and culture

Samples of decidua were obtained by vaginal curettage from elective vaginal termination of first trimester pregnancies (6–11 weeks) from healthy women aged 20–30 years. None of the abortions was pharmacologically induced. We excluded women receiving any medication or with infectious, autoimmune or other systemic or local diseases. Samples were obtained at the Clínica El Sur in Málaga and the Clínica Ginegranada in Granada.

To obtain fresh DSCs, we used a protocol similar to that reported by Montes *et al.* (1995). Decidual tissue was washed in PBS and minced between two scalpels in a small volume of Roswell Park Memorial Institute medium (RPMI) 1640 with 10% fetal calf serum (FCS). The cell suspension was incubated with 5 mg/ml collagenase V (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C. This preparation was filtered through sterile gauze, washed and suspended in the culture medium. The cell suspension was centrifuged at 650g for 20 min over a Ficoll-Histopaque 1077 density gradient (Sigma-Aldrich). Cells were then collected from the interphase and washed. This suspension, containing mainly DSCs and leukocytes, was incubated for 1 h in complete RPMI with 10% FCS to allow macrophages and granulocytes to adhere to the flask. The supernatant was incubated overnight so that DSC adhered to the plate. Lymphocytes in the supernatant were discarded, leaving a highly purified population of DSCs free of granulocytes and macrophages. Purity was confirmed by the detection of co-expression of CD10 and CD29, and the absence of CD45 (which identifies leukocytes) and cytokeratin (which detects epithelial cells and trophoblast) in 95–100% of DSC (Garcia-Pacheco *et al.*, 2001; Kimatrai *et al.*, 2003, 2005). Only preparations with >95% viable cells as determined by trypan blue exclusion were used.

To establish DSC lines, purified DSCs were maintained and expanded in Opti-MEM medium (Invitrogen, Paisley, UK) with 3% FCS for 8–12 weeks. In this low serum-containing media, DSC showed stable antigen phenotype and functional activities (Oliver *et al.*, 1999; Garcia-Pacheco *et al.*, 2001; Kimatrai *et al.*, 2003, 2005).

Follicular dendritic cell isolation and culture

Tonsil samples were obtained by tonsillectomy from patients with tonsillitis at the Hospital Universitario Virgen de las Nieves (Granada, Spain). Patients (3–10 years old) were in complete remission before surgery. Informed consent was obtained from the parents or guardians of each patient. Follicular dentritic cell (FDC) lines were established as previously described (Munoz-Fernandez *et al.*, 2006).

Lymphocyte isolation and culture

To extract decidual lymphocytes, samples of decidua were fragmented, minced in a small volume of RPMI 1640 and then cut and sieved through a 53-μm sieve (Gallenkamp, Loughborough, UK) to obtain cell suspensions. The suspensions were washed with PBS and centrifuged on Ficoll-Histopaque 1077. The cells were collected from the interface, suspended in complete culture medium (RPMI 1640, 10% FCS, 100 U/ml penicillin and 50 g/ml gentamicin) and incubated for 2 h at 37°C in an atmosphere of 5% CO_2/95% air to allow adherent cells to attach to the plastic. The supernatant-containing decidual lymphocytes were then collected and purity was confirmed by flow cytometry (>90% $CD45^+$).

To obtain peripheral blood lymphocytes, blood samples were taken from healthy volunteers aged 20–35 years. Peripheral blood mononuclear cells were prepared by Ficoll-Histopaque density gradient centrifugation and adherent monocytes were depleted by culture on plastic dishes for 1 h at 37°C. The purity of lymphocytes was determined by flow cytometry (>95% $CD45^+$).

Ethical approval

The research and ethics committee of the Hospital Universitario San Cecilio in Granada approved this study. Informed consent was obtained from all patients.

Tumor cell lines

The human leukemia T-cell line Jurkat and the human breast carcinoma cell line SKBR3 were maintained in culture in RPMI 1640 medium with 10% FCS, L-glutamine, penicillin and streptomycin at 37°C in a humidified incubator with 5% CO_2 and 95% air.

Reagents and antibodies

P4, 8-bromo-cAMP, β-estradiol, PRL, decitabine, doxorubicin, LY294002, nocodazole and mouse anti-β-actin were purchased from Sigma-Aldrich. Bisindolylmaleimide and SP600125 were obtained from Calbiochem (Darmstadt, Germany). Human recombinant TRAIL was prepared as previously described (MacFarlane *et al.*, 1997). CH11 mAb (IgM), which reacts with CD95, was from Upstate Biotechnology (Lake Placid, NY, USA). Anti-PRL receptor antibody and TGF-β were obtained from R&D Systems (Minneapolis, MN, USA). Anti-caspase-3 polyclonal antibody was obtained from Stressgen Bioreagents (Ann Harbor, MI, USA) and used at a dilution of 1:1000.

Detection of apoptotic cells

Hypodiploid apoptotic cells were detected by flow cytometry according to a published procedure (Gong *et al.*, 1994). Briefly, cells were washed with PBS, fixed in cold 70% ethanol and then stained with propidium iodide during treatment with RNase. Quantitative analysis of sub-G1 apoptotic cells was carried out in an FACScan cytometer.

Decidualization of DSC

To induce decidualization, cells cultured to 70% confluence were treated with 300 nM P4 and 500 μM 8-bromo-cAMP for 28 days. The culture medium was changed every 3–4 days and these factors were readded. The decidualization of DSC was assessed by changes in cell morphology and PRL secretion.

Prolactin analysis

The concentration of PRL in supernatants from decidualized DSC was determined by ELISA (Roche, Basel, Switzerland, Catalog Number: 032030931 90) according to manufacturer's instructions.

Immunoblots detection of proteins

For detection of cytosolic proteins, cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–Cl, 1% NP-40) for 30 min. Proteins of cytosolic supernatants were resolved on 10% SDS-PAGE gels and detected as reported previously (Ruiz-Ruiz and Lopez-Rivas, 1999).

Statistical analysis

All experiments were performed in duplicate and repeated at least three times. We used Shapiro–Wilk's test to verify the normality of the data and Levene's test to test the equality of variances. The data were analyzed with the one-way and two-way analysis of variance (ANOVA). In case of data with no equality of variances in the one-way ANOVA, we used Welch's test. In all cases in which ANOVA was significant, multiple comparison methods and contrasts methods were used. Values of $P < 0.05$ were considered significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

DSCs are resistant to different pro-apoptotic stimuli

Our previous results demonstrated that DSC expressed CD95 and TRAIL-Receptor 2 (TRAIL-R2), although they were resistant to apoptosis mediated by both death receptors in short-term experiments (Blanco *et al.*, 2009). Here, we verified that DSC remained resistant to CD95- and TRAIL-R2-mediated apoptosis after prolonged incubation with either an agonistic anti-CD95 antibody or recombinant TRAIL (Fig. 1). To determine whether DSCs exhibit an apoptosis-resistant phenotype, we analyzed the response of these cells to common inducers of apoptosis. Specifically, we tested the DNA-methyltransferase inhibitor decitabine, the genotoxic drug doxorubicin, the microtubule depolymerizing agent nocodazol, the PI3K inhibitor LY294002, the protein kinase C inhibitor bisindolylmaleimide and the c-Jun N-terminal kinase inhibitor SP600125. Working concentrations of these compounds were chosen according to previous reports (Hoshino *et al.*, 2001; Morales *et al.*, 2007; Moon *et al.*, 2008; Pajak *et al.*, 2008; Ruiz-Magaña *et al.*, 2012). Interestingly, DSC showed resistance to all apoptotic drugs after 48 h of incubation (Fig. 1). In this set of experiments, we used Jurkat cells as a positive control.

DSC undergo apoptosis during decidualization in culture

We then examined the effect of decidualization on the viability of DSC. To this end, DSCs were incubated with P4 and 8-bromo-cAMP for

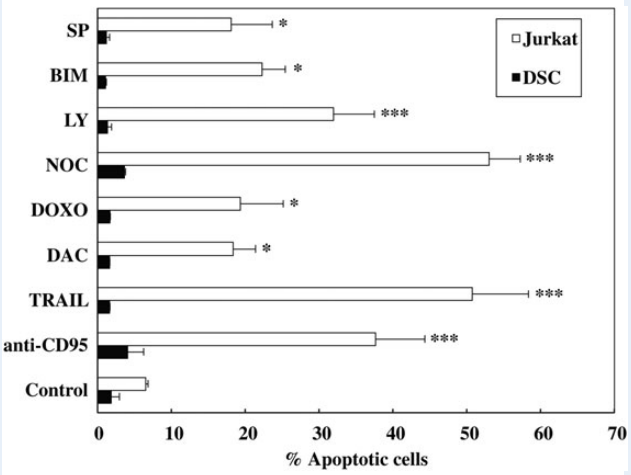

Figure 1 Induction of apoptosis in DSCs in response to treatment with different stimuli. DSCs were treated without (Control) or with TRAIL (250 ng/ml) or anti-CD95 (100 ng/ml) for 96 h, or with decitabine (DAC, 5 μM), doxorubicin (DOXO, 200 ng/ml), nocodazol (NOC, 400 ng/ml), LY294002 (LY, 10 μM), bisindolylmaleimide (BIM, 6 μM) or SP600125 (SP, 10 μM) for 48 h. Jurkat cells were incubated for 24 h in the same conditions, except for TRAIL (100 ng/ml) and anti-CD95 (5 ng/ml). The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Error bars show the standard deviation for three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control cells.

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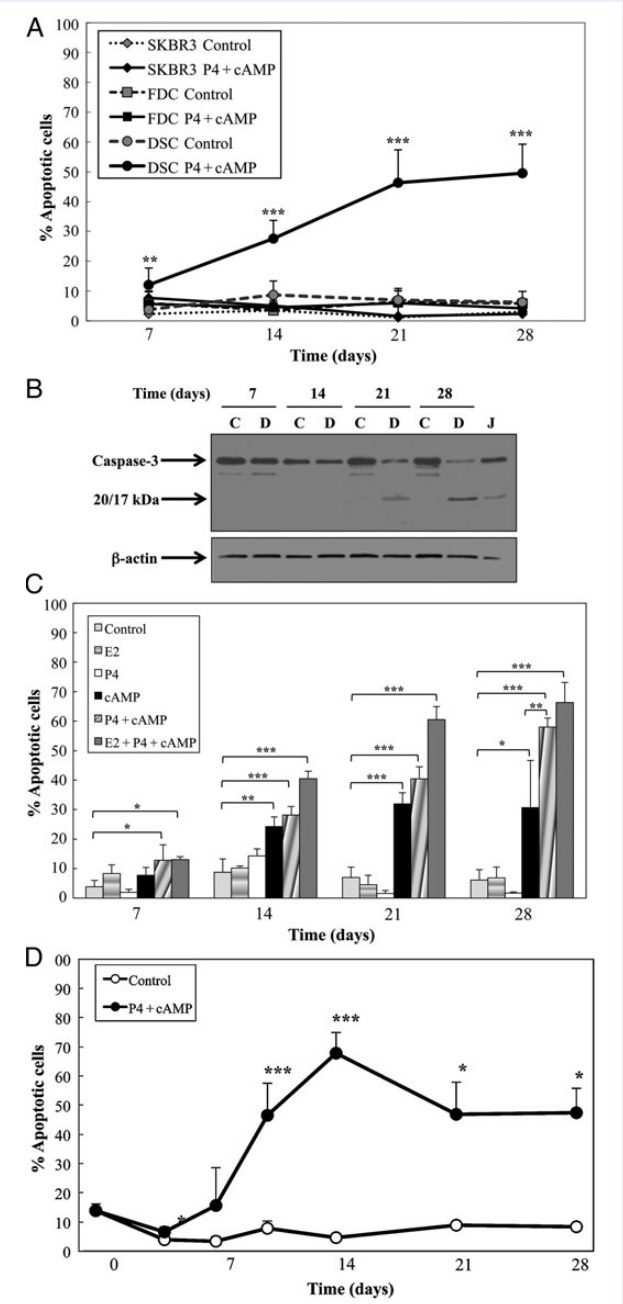

Figure 2 Induction of apoptosis upon decidualization in culture. DSC (**A** and **B**), follicular dendritic cells (FDCs) and SKBR3 cells (A) and fresh DSC (**D**) were untreated (Control) or decidualized with P4 (300 nM) and 8 bromo-cAMP (500 μM) for 28 days. (**C**) DSCs were incubated without (Control) or with E_2 (30 nM), P4 (300 nM) and 8-bromo-cAMP (500 μM) alone or in combination for 28 days. In (A, C and D), sub-G1 apoptotic cells were analyzed by flow cytometry every 7 days. In (B), activation of caspase-3 was determined by western blot in untreated control (C) and decidualized (D) DSC. Apoptotic Jurkat cells treated with TRAIL were used as a positive control (J). β-actin was used as a control of loaded protein. Bands at 20/17 kDa represent the active form of caspase-3. Error bars show the standard deviation for three (FDC, SKBR3, DSC in C and fresh DSC in D) and six (DSC in A) independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus controls.

28 days and apoptosis was determined every 7 days. A time-dependent increase in apoptosis was observed from the first week of treatment (Fig. 2A). To rule out the possibility of a toxic side effect of the decidualization agents, we also analyzed the induction of apoptosis in two other models: FDC, which are related to DSC (Munoz-Fernandez *et al.*, 2006, 2012), and the apoptosis-sensitive breast cancer cell line SKBR3 (Morales *et al.*, 2007). As shown in Fig. 2A, there was no apoptosis in either FDC or SKBR3 cells after treatment with P4 and 8-bromo-cAMP at any time point. We further confirmed the induction of apoptosis in DSC by determining the activation of caspase-3 (Fig. 2B). The active caspase-3 fragment was detected at 21 and 28 days of decidualization. Moreover, a decrease in the levels of intact caspase-3, which indicates its activation, was observed in decidualized DSC since Day 14.

In order to determine the decidualization factors required for apoptosis induction, DSCs were incubated with P4 and 8-bromo-cAMP either alone or in combination for 28 days and apoptosis was again analyzed every 7 days. In addition, we studied the effect of E_2, a hormone present during pregnancy which may influence DSC decidualization (Ramathal *et al.*, 2010). Neither P4 nor E_2 alone or in combination induced cell death (Fig. 2C and data not shown). In contrast, DSC incubated with 8-bromo-cAMP underwent significant apoptosis after 14, 21 and 28 days (Fig. 2C). As shown in Fig. 2A, cell death was evident from the first week of incubation when 8-bromo-cAMP was combined with P4, although apoptosis induced by this combination was only significantly higher than that induced by 8-bromo-cAMP alone at Day 28. The addition of E_2 to treatment with 8-bromo-cAMP and P4 further increased the percentage of apoptotic DSC (Fig. 2C).

To study DSC in a context closer to the physiological setting, we analyzed the induction of apoptosis in freshly isolated DSC incubated with P4 and 8-bromo-cAMP for 28 days. A decrease in the percentage of apoptotic cells was observed on Day 4 relative to Day 0, regardless of the presence or absence of P4 and 8-bromo-cAMP. However, we found an increase in apoptosis in response to treatment with both factors from Day 7, being significant from Day 10 onward.

Decidualized DSC secrete apoptosis-inducing factors

Decidualization of DSCs in culture was achieved by removing the medium and replacing it with fresh medium supplemented with P4 and 8-bromo-cAMP every 3 or 4 days. To ascertain the need for a continuous supply of P4 and 8-bromo-cAMP for apoptosis induction, DSCs that had been treated with these factors for 16 days (Day 0) were washed and incubated in fresh medium with or without P4 and 8-bromo-cAMP for a further 4 days. Although the percentage of apoptotic cells did not change upon reincubation with P4 + 8-bromo-cAMP, it was moderately decreased after withdrawal of these factors (Fig. 3A). For comparison, apoptosis was also evaluated in 16-day decidualized DSC maintained for a further 4 days without replacing the medium. Interestingly, these cultures showed the highest level of apoptosis 4 days later regardless of the readdition of P4 and 8-bromo-cAMP (Fig. 3A, old medium (OM)).

To ascertain whether continuous treatment of DSC with P4 and 8-bromo-cAMP induced the secretion of soluble factors capable of triggering apoptosis, non-differentiated DSCs were incubated with conditioned medium from DSC treated for 16 days with P4 and 8-bromo-cAMP (CM-D). After 4 days of incubation under these conditions, ~40% of undifferentiated DSC showed apoptosis (Fig. 3B). To rule

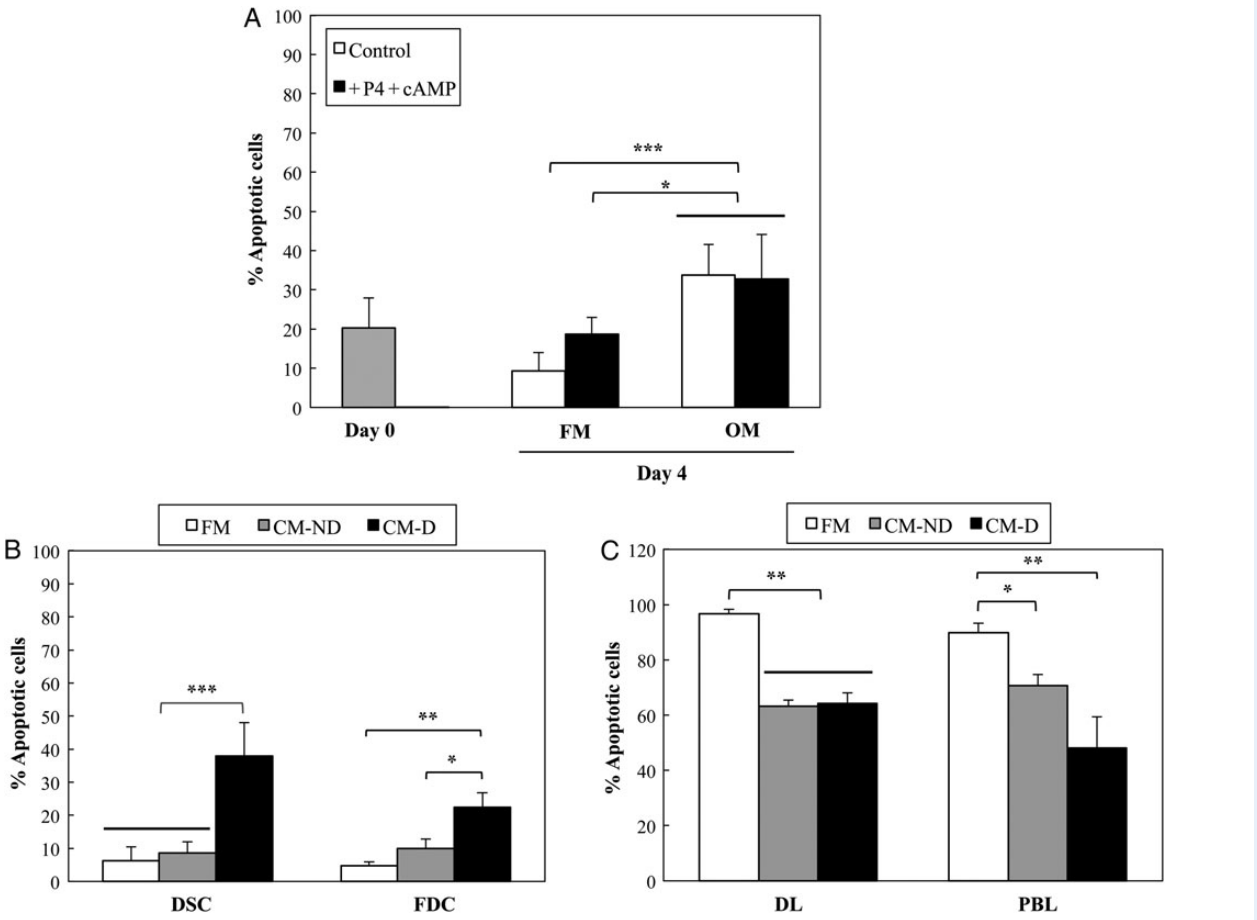

Figure 3 Induction of apoptosis by conditioned medium from decidualized DSC. (**A**) DSCs were decidualized with P4 and 8-bromo-cAMP for 16 days (Day 0). Then they were incubated without (Control) or with the readdition of P4 and 8-bromo-cAMP (+P4 + cAMP) either in fresh medium (FM) or without replacing the medium (OM) for another 4 days (Day 4). (**B**) DSC and FDC were incubated for 4 days with fresh medium (FM), conditioned medium from non-differentiated DSC (CM-ND) or conditioned medium from 16-day decidualized DSC (CM-D). (**C**) Decidual lymphocytes (DL) and peripheral blood lymphocytes (PBL) were incubated as in (B). The percentage of apoptotic cells was measured by flow cytometry. Error bars show the standard deviation for three (FDC, DL and PBL) and six (DSC) independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

out a possible effect of the exhaustion of nutrients in the supernatants, control cells were incubated with supernatants derived from cultures of non-decidualized DSC (CM-ND), which have a higher proliferative activity than decidualized DSC (data not shown). As expected, DSC viability in this case was similar to that of cells cultured with fresh medium (Fig. 3B). We also ruled out a potential direct effect of residual P4 and 8-bromo-cAMP in the supernatants, because DSC began to undergo apoptosis in response to these factors at later time points (7 days, Fig. 2). These experiments were also carried out in FDC. As shown in Fig. 3B, conditioned medium from decidualized DSC induced apoptosis in FDC, whereas no significant apoptosis was observed in control FDC incubated with supernatants from non-differentiated DSC. We previously showed that FDC did not undergo apoptosis when incubated with P4 and 8-bromo-cAMP (Fig. 2A), a finding that further ruled out an effect due to the residual presence of these factors in the supernatants.

Decidual lymphocytes represent an important cell population in the decidua. We recently showed that DSCs produce soluble factors of importance for the survival of decidual lymphocytes as well as peripheral lymphocytes in culture (Blanco *et al.*, 2009). Accordingly, we analyzed the effect of decidualized DSC supernatants on lymphocyte viability. Strikingly, upon decidualization, DSCs were still able to secrete factors that protect decidual and peripheral lymphocytes from spontaneous apoptosis in culture, even more significantly than non-decidualized DSCs (Fig. 3C).

Lack of relationship between apoptosis and prolactin secretion in decidualized DSC

An important marker of the decidualization process is the production of PRL (Dunn *et al.*, 2003). To test PRL secretion by decidualized DSCs under our culture conditions, DSCs were incubated with 8-bromo-cAMP, P4 and E_2, either alone or in combination, for 28 days and PRL concentration in the supernatants was measured every 7 days. Incubation with either P4 or E_2 induced weak PRL secretion on Days 21 and 28, whereas 8-bromo-cAMP, either alone or combined

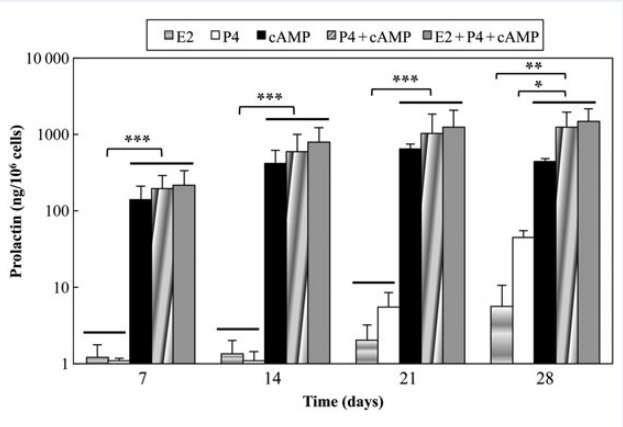

Figure 4 PRL secretion by decidualized DSC. DSCs were treated with E_2 (30 nM), P4 (300 nM) or cAMP (500 μM) alone or in combination for 28 days. The secretion of PRL was determined by ELISA every 7 days. Error bars show the standard deviation for three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

with P4 with or without E_2, induced a large increase in PRL production at all time points (Fig. 4).

We investigated the involvement of PRL in apoptosis induction during decidualization. DSC decidualized for 16 days were incubated with exogenously added PRL at a concentration similar to that in the cultures of decidualized cells. After 4 days of incubation, no increase in apoptosis was observed in cells treated with PRL in fresh medium compared with untreated control cells (Fig. 5A). Likewise, the addition of PRL did not increase the percentage of apoptotic cells in decidualized DSC cultures either incubated with P4 plus 8-bromo-cAMP or maintained in their conditioned medium (Fig. 5A). Similar experiments were done with non-differentiated DSC. Again, treatment for 4 days with PRL did not affect the viability of DSC under any of the culture conditions (Fig. 5B). We then evaluated the effect on apoptosis of PRL when combined with other factors known to be produced by decidualized cells, such as TRAIL, TGF-β1 and CD95L (Popovici *et al.*, 2000; Stoikos *et al.*, 2008; Garrido-Gomez *et al.*, 2011). The DSC did not undergo significant apoptosis in response to any combination tested after 4 days of incubation (Fig. 5C). Finally, we ruled out the involvement of PRL in DSC apoptosis during decidualization by blocking the activity of this hormone with a PRL

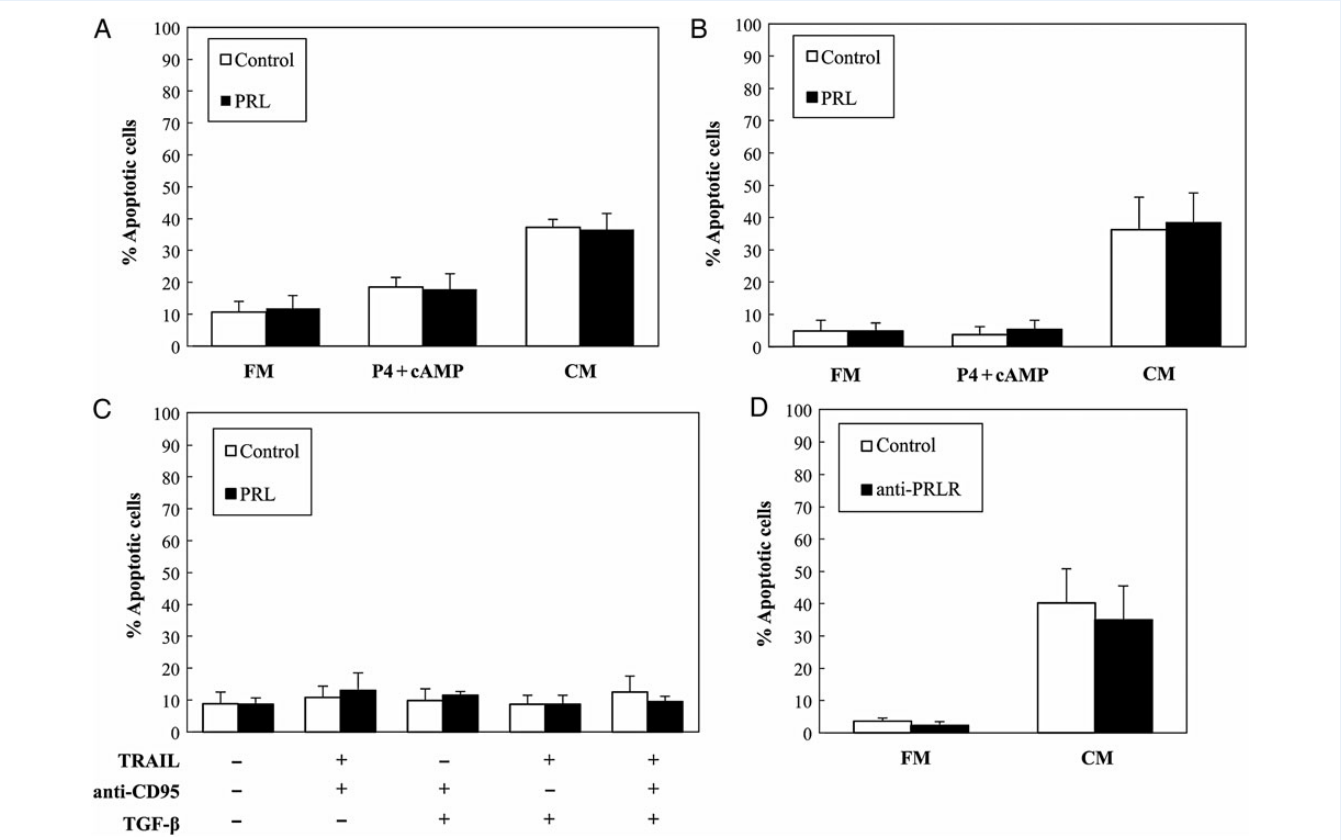

Figure 5 Contribution of secreted PRL to the induction of apoptosis in DSC. Sixteen-day decidualized DSC (**A**) and non-decidualized DSC (**B**) were incubated with fresh medium (FM), P4 and 8-bromo-cAMP (P4 + cAMP) or conditioned medium from decidualized DSC (CM) in the absence (Control) or in the presence of PRL (200 ng/ml) for 4 days. (**C**) DSCs were treated for 4 days with different combinations of TRAIL (250 ng/ml), anti-CD95 (100 ng/ml) or TGF-β (4 ng/ml), as indicated in the figure, in the absence (Control) or in the presence of PRL (200 ng/ml). (**D**) DSCs were incubated without (Control) or with a PRL receptor antibody (anti-PRLR, 9 μg/ml) either in fresh medium (FM) or in conditioned medium from 16-day decidualized DSC (CM) for 4 days. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Error bars show the standard deviation for three independent experiments.

receptor antibody. Non-differentiated DSCs were incubated with conditioned medium from decidualized DSC; as shown in Fig. 5D, the neutralizing antibody against PRL receptor did not significantly reduce supernatant-induced apoptosis.

Discussion

The human endometrium is a highly dynamic tissue that undergoes continuous cycles of remodeling. Apoptosis plays a key role in the tissue restructuring and maintenance of cellular homeostasis throughout the menstrual cycle (Kokawa *et al.*, 1996). In addition, the formation of the decidua during pregnancy involves DSC proliferation, differentiation and apoptosis (Dunn *et al.*, 2003; Mikhailov, 2003). This cell death process is thought to limit the lifespan of DSC, allowing placental expansion and development. Consequently, the regulation of placental apoptosis plays a major role in the normal physiology of pregnancy, although the factors involved are not yet known (Gu *et al.*, 1994; Mikhailov, 2003).

Here we have shown that DSC underwent apoptosis during decidualization, although they were highly resistant to a variety of apoptotic stimuli. This implies that DSC death may be a strictly regulated process that occurs only under conditions similar to those found in pregnancy. Treatment with 8-bromo-cAMP alone induced apoptosis in DSC, whereas the same effect was not observed upon treatment with either P4 or E_2 (Fig. 2C). These data suggest that decidual apoptosis during pregnancy may occur in response to factors produced by the endometrium, such as relaxin, prostaglandin E_2 and corticotropin-releasing hormone, which induce an increase in the intracellular concentrations of cAMP. Nevertheless, apoptosis was greater when DSCs were incubated with 8-bromo-cAMP and P4 with or without E_2, particularly at later time points. Similarly, we showed that PRL secretion, an important marker of decidualization, depended essentially on stimulation by cAMP, although sustained production required the presence of P4 (Fig. 4, Day 28). These results are in agreement with previous data demonstrating that elevated intracellular levels of cAMP are essential for decidualization to begin and for DSC to become sensitized to P4; although, sustained decidualization requires both cAMP and P4 (Gellersen and Brosens, 2003). The role of E_2 is likely to be to improve the response to P4 as it has been reported to up-regulate the expression of P4 receptor (Schultz *et al.*, 2005). On the whole our results indicate that the apoptosis of decidualized DSC depends on the same factors that promote the decidualization process. Interestingly, we also observed that P4 and E_2 did not affect the proliferation of DSC, whereas cAMP alone or in combination with these hormones reduced cell proliferation (data not shown), further reinforcing the role of cAMP in the changes that occur in DSC during decidualization. Several transcription factors such as p53, FOXO-1 and TWIST1 have been reported to increase in response to cAMP and decidualization. In addition to participating in the differentiation process, these factors may play a role in the apoptotic cell death of decidualized DSC (Gellersen and Brosens, 2003; Pohnke *et al.*, 2004; Schroeder *et al.*, 2011).

It is well known that DSC decidualization involves not only a change in the expression of proteins related to metabolism, the cytoskeleton and apoptosis, but also the secretion of cytokines and other factors such as death receptor ligands (Popovici *et al.*, 2000; Garrido-Gomez *et al.*, 2011) which may be responsible for the apoptotic effect of decidualized DSC-conditioned medium (Fig. 3). As noted, PRL is one of the major proteins secreted by DSC during decidualization. Although proliferative functions have been ascribed to PRL, this hormone also has an apoptotic effect in some models such as the androgen-responsive prostatic adenocarcinoma cell line LNCaP and the corpus luteum in cyclic rats (Gaytan *et al.*, 2000; Giuffrida *et al.*, 2010). Our results suggest that PRL is not directly involved in the induction of apoptosis in human DSC. The kinetic correlation observed between PRL secretion and apoptosis induction in DSC lines as well as in fresh DSC (Figs 2C and 4 and data not shown) is probably an indication that similar signaling or transcription factors regulate both events during decidualization. In this connection, Tessier *et al.* (2001) have suggested an anti-apoptotic role for PRL in rat decidua. However, in our model the addition of PRL did not reduce apoptosis in decidualized DSC incubated with their conditioned medium (Fig. 5A) (Moulton, 1994). The discrepancies between our data and those of other authors may be due to the use of different experimental models (human versus rat cells).

We also studied the apoptotic effect of other factors known to be secreted by decidualized DSC, but found that undifferentiated DSCs were highly resistant to all of them (Figs 1 and 5C). This result rules out a major contribution of these factors to the induction of apoptosis during decidualization. Our findings of resistance to TRAIL and anti-CD95 agonistic antibody are in agreement with previous reports (Fluhr *et al.*, 2007, 2009). The role of TRAIL and CD95L at the maternal–fetal interface was recently suggested to lie in the modulation of the endometrial and decidual environment, which favors embryo implantation and pregnancy (Fluhr *et al.*, 2009, 2011). Regarding TGF-β, this cytokine has been described as an inducer of apoptosis in the rat decidua. The resistance observed in our human DSC again suggests differences between species (Moulton, 1994). Other decidual factors have also been associated with cell death. Parathyroid hormone-like hormone, produced by DSC during decidualization, induces stromal cell apoptosis and represses uterine stromal cell differentiation (Sherafat-Kazemzadeh *et al.*, 2011). These observations are interesting in that they suggest an inverse correlation between apoptosis and decidualization. Chobotova *et al.* (2005) also described an inverse association between these two processes in response to heparin-binding epidermal growth factor, which seems to induce decidualization while improving the survival of endometrial stromal cells.

FDCs have been reported to share some phenotypical and functional features with DSC (Munoz-Fernandez *et al.*, 2006, 2012). Here we demonstrate that FDC did not undergo apoptosis when cultured with P4 and 8-bromo-cAMP, although their morphology changed and they produced PRL (Munoz-Fernandez *et al.*, 2012). However, FDC, like undifferentiated DSC, were sensitive to apoptosis induced by decidualized DSC-conditioned medium, further indicating that decidualized DSCs secrete factors able to trigger apoptosis in DSC and related cells (such as FDC). Intriguingly, the survival of decidual and peripheral lymphocytes improved after incubation with decidualized DSC supernatants, as was previously shown with supernatants from non-decidualized DSC (Blanco *et al.*, 2009). It is to be anticipated that very different types of cells (hematopoietic and stromal cells) behave in a different way in response to a conditioned medium that may contain diverse factors. Several cytokines and biologically active molecules produced by DSC regardless of their differentiation stage may be responsible for this protective effect (Engert *et al.*, 2007; Popovici *et al.*, 2000).

Taken together, our results suggest that cAMP induces the decidualization of DSC as well as the production of soluble factors that induce apoptosis in these cells in an autocrine or paracrine manner. Experiments

with fresh DSC confirmed the induction of cell death during decidualization, but with faster kinetics than in cultured DSC lines. The same signaling pathways activated in response to cAMP may lead to differentiation and apoptosis in DSC; however, at present we do not know whether they are related or independent processes. Further studies are needed to ascertain the molecular mechanism that regulates these two phenomena.

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Authors' roles

E.L.-D. executed the experiments, collected tissues and prepared the figures. M.J.R.-M. and R.M.-F. collected tissues and assisted in the experiments. F.R. performed the statistical analysis. E.G.O. and C.R.-R. designed the study and drafted the manuscript.

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Conflict of interest

None declared.

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