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Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis)

Maurizio Pesce[1], Maria Grazia Farrace[2], Mauro Piacentini[2], Susanna Dolci[1] and Massimo De Felici[1,*]

[1]Dipartimento di Sanità Pubblica e Biologia Cellulare and [2]Dipartimento di Biologia, Università di Roma 'Tor Vergata', Via O. Raimondo, 8, 00173 Roma, Italy

*Author for correspondence

SUMMARY

Proliferating primordial germ cells (PGCs) isolated from mouse embryos soon after their arrival in the genital ridges would only survive in vitro at temperature of less than 30°C (De Felici, M. and McLaren, A. (1983). *Exp. Cell. Res.* 144, 417-427; Wabik-Sliz, B. and McLaren, A. (1984). *Exp. Cell. Res.* 154, 530-536) or when co-cultured on cell feeder layers (Donovan, P. J., Stott, D., Godin, I., Heasman, J. and Wylie, C. C. (1986). *Cell* 44, 831-838; De Felici, M. and Dolci, S. (1991). *Dev. Biol.* 147, 281-284).

In the present paper we report that mouse PGC death in vitro occurs with all the hallmarks of programmed cell death or apoptosis. We found that after 4-5 hours in culture many PGCs isolated from 12.5 dpc fetal gonads assumed a nuclear morphology and produced membrane bound fragments (apoptotic bodies) typical of apoptotic cells. In addition, PGCs in culture accumulated high level of tissue transglutaminase (tTGase; an enzyme that is induced and activated during apoptosis) and showed extensive degradation of DNA to oligonucleosomal fragments, which is characteristic of apoptosis. The physiological relevance of this mechanism of PGC death is supported by the finding that some PGCs undergoing apoptosis, as revealed by the high level of tTGase expression, were detected in the embryo. Most importantly, we show that the addition of stem cell factor (SCF) or leukemia inhibitory factor (LIF) to the culture medium, two cytokines known to favour PGC survival and/or proliferation in vitro, markedly reduced the occurrence of apoptosis in PGCs during the first hours in culture. These last results suggest a novel mechanism by which these two cytokines may affect the in vitro as well possibly in vivo development of mammalian PGCs.

Key words: primordial germ cells, stem cell factor, leukemia inhibitory factor, apoptosis

INTRODUCTION

Mutations in the murine *White spotting* (*W*) and *Steel* (*Sl*) genes, which respectively encode the c-*kit* tyrosine kinase receptor and its ligand (known as steel factor, SF, stem cell factor, SCF, or mastocyte growth factor, MGF), result in deficiencies of primordial germ cells (as well of mast cells, hemopoietic stem cells and melanocytes) (Mintz and Russell, 1957). Whereas the c-*kit* gene is expressed in primordial germ cells (PGCs; Orr-Urtreger et al., 1990), the SCF gene is expressed along their migratory pathway and in the genital ridges (Matsui et al., 1990). Recent in vitro studies have proved that SCF is essential for the survival and/or proliferation of mouse PGCs (Dolci et al., 1991; Matsui et al., 1991; Godin et al., 1991). Leukemia inhibitory factor (LIF), a cytokine with a broad range of effects on different cell types, has also been shown to affect PGC survival in culture (De Felici and Dolci, 1991).

A key aspect of the action operated by trophic hormones and other growth factors during development is the prevention of programmed cell death (apoptosis). Apoptosis is the physiological process of cell death leading to the controlled elimination of single cells from the midst of a viable tissue. This fundamental process plays a key role in tissue remodelling occurring during embryonic development and metamorphosis and in the regulation of the cell number in normal tissue (Fesus et al., 1991; Arends and Wyllie, 1991). Although the molecular mechanisms leading to apoptosis are not fully characterized, requirement of RNA and protein synthesis indicate that expression of specific genes is necessary for this physiological death. Some morphological and biochemical features common to apoptotic cells have been established. The chromatin undergoes typical condensation which is paralleled by its fragmentation at internucleosomal sites by an as yet unidentified Ca^{2+}-dependent endonuclease (Shi et al., 1989; Wyllie et al., 1984; Smith et al., 1989); the cytoplasm also becomes condensed and an active membrane blebbing leads to the formation of several membrane-bound cellular fragments (apoptotic bodies; Fesus et al., 1991; Arends and Wyllie, 1991), containing highly cross-linked protein polymer (Fesus et al., 1989) catalyzed by an intracellular transglutaminase, tissue transglutaminase (tTGase), induced and activated specifically in apoptotic cells (Folk, 1980; Knight et al., 1991; Fesus et al.,

1989; Piacentini et al., 1991). Overexpression of tTGase in BALB-C 3T3 fibroblasts determines in transfected cells the cytoplasmatic changes (blebbing, condensation) typical of apoptosis (Gentile et al., 1992).

In the present paper we show that proliferating PGCs purified from mouse embryos rapidly undergo apoptosis in vitro and that the soluble forms of SCF and LIF temporarily prevent this process. This suggests a novel role for these cytokines and apoptosis in regulating the establishment of germ cell population in the mammalian embryo.

MATERIALS AND METHODS

Isolation and culture of primordial germ cells

Embryos were obtained from CD-1 mice (Charles River, Italy) on the 13th-18th day of pregnancy (12.5-17.5 days post coitum). From 12.5 dpc onwards gonads were sexed by their characteristic appearance. Primordial and fetal germ cells with minimal somatic cell contamination (purity about 70%-80%) were isolated from gonads by EDTA-mechanical treatment according to the method of De Felici and McLaren (1982). Staining of PGCs by alkaline phosphatase according to the method described by De Felici and Dolci (1989) was always used as the criterion of identification to estimate the purity of the PGC population. Cultures were maintained at 37°C in 5% CO_2 for the indicated times, using a modified MEM (De Felici and Dolci, 1991), supplemented with 5% horse serum and 2.5% heat-inactivated fetal calf serum (Flow), in 8-well Lab-Tek chamber slides coated with a thin layer (approximately 0.5-1 mm) of reconstituted basement membrane (Matrigel, Collaborative Research). Leukemia Inhibitory Factor (LIF, human recombinant) and Stem Cell Factor (SCF, mouse recombinant) were purchased from Genzyme. Cycloheximide (Sigma) was freshly dissolved at 1 mg/ml in distilled water and diluted with the culture medium immediately before use.

Light and SE microscopy

Semithin sections of purified PGCs in culture were prepared as follows. PGCs were collected in 1.5 ml Eppendorf tubes before being centrifuged, washed and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 hour. After thoroughly wash in cacodylate buffer, PGCs were post-fixed in 1% aqueous osmium tetroxide for 1 hour, dehydrated in a series of graded ethanol solutions, cleared in toluol and embedded in Epon.

For SEM observations PGCs were cultured on glass coverslips coated with Matrigel before being fixed and post-fixed as reported above. The specimen was then washed in cacodylate buffer, dehydrated in ethanol, and dried by the critical-point dryer method. After coating with gold the specimen was viewed under an Hitachi SEM.

Immunolabeling for tTGase of PGCs in culture was performed using affinity-purified rabbit IgG raised against soluble tTGase of human red blood cells as previously reported (Piacentini et al., 1991).

Genital ridges from 12.5-13.5 dpc embryos were fixed in 4% paraformaldehyde for 1 hour and, after embedding in Tissue-tek OCT compound, frozen in liquid nitrogen. Serial sections (8 or 10 μm) were cut in a cryostat and stained for alkaline phosphatase to identify germ cells (De Felici and Dolci, 1989) or incubated in polyclonal antibodies against soluble tissue transglutaminase (tTGase) to detect apoptotic cells (Piacentini et al., 1991).

DNA extraction, labeling and electrophoresis

Approximately 2×10^4 purified PGCs were lysed overnight at 37°C in 1 ml of a solution containing 200 mM NaCl, 20 mM EDTA, 40 mM Tris-HCl (pH 8.00), 0.9% SDS and 400 μg/ml proteinase K. The cell lysates were precipitated with saturated NaCl (6 M) and 100% ethanol (2:1, v/v) added to supernatants to precipitate DNA. The samples were resuspended in H_2O and stored at −20°C. For autoradiographic analysis, DNA samples (0.4 μg) were labeled at the 3′-end with [α^{32}P]dideoxy-ATP (4000 Ci/mmol) by incubation for 60 minutes at 37°C in the presence of 25 U terminal transferase (Boehringher-Mannheim). Labeled DNA was electrophoresed (0.4 μg/lane) by a 2% agarose gel. Gels were dried in a slab-gel dryer for 2 hours and exposed to Kodak X-ray films for 1-3 hours at −80°C.

RESULTS

Occurrence of apoptosis in primordial germ cells as determined by light and SE microscopy

When observed in semithin sections, about 50% of 12.5 dpc PGCs cultured for 4-5 hours showed typical apoptotic morphology. Varying degrees of cytoplasmic condensation and the development of sharply circumscribed masses of compacted chromatin is shown in Fig. 1. Moreover, budding of affected cells to produce apoptotic bodies was frequently seen (Fig. 1). PGC fragmentation resulting in the formation of numerous apoptotic bodies was also observed by SEM (Fig. 2).

Post-mitotic germ cells isolated from 16.5-17.5 dpc fetal gonads, which survive for some days in culture (De Felici

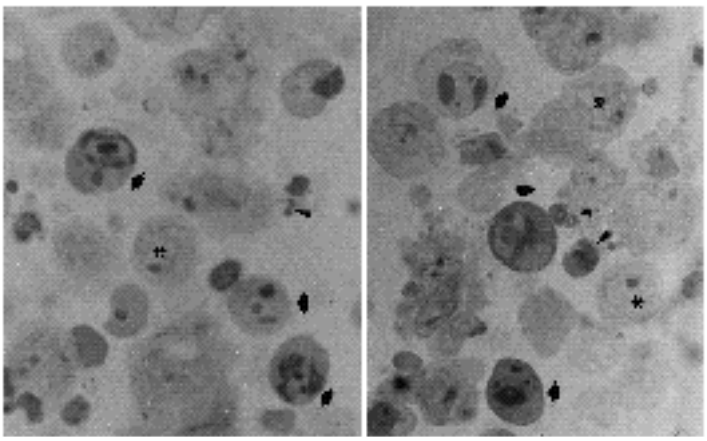

Fig. 1. Apoptotic morphologies of 12.5 dpc PGCs cultured for 4-5 hours as seen in semithin sections. Note characteristic chromatin condensation (arrows) and apoptotic bodies (arrowheads). Some normal non-apoptotic PGCs are indicated by asterisks.

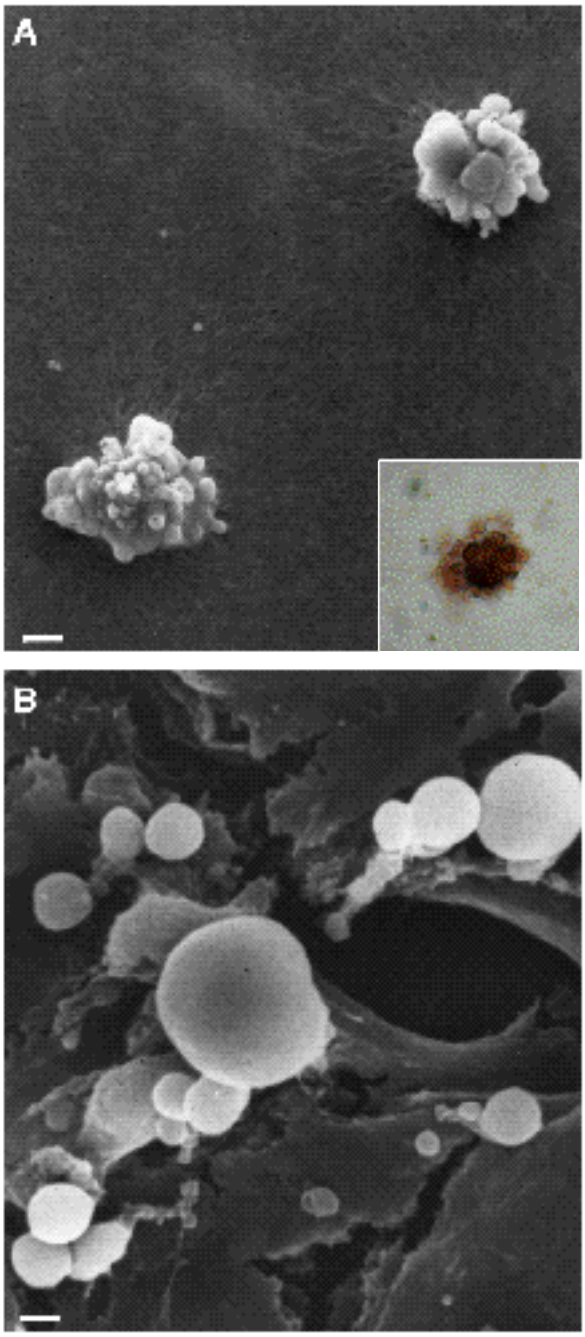

Fig. 2. SEM micrographs of 12.5 dpc PGCs forming numerous apoptotic bodies in culture. (A) Initial membrane blebbing. (B) Final cell fragmentation in apoptotic bodies. Insert, formation of apoptotic bodies in a tTGase labeled PGC. Bar approximately 4 μm (A) and 2 μm (B).

and McLaren, 1983), did not show such apoptotic morphology (not shown).

Immunolocalization of tTGase protein

Since it is well established that apoptotic cells usually express high levels of tTGase (Fesus et al., 1991), we carried out an immunolocalization of this enzyme in 12.5 and 16.5 dpc germ cells after different culture times. The immunostaining revealed that while PGCs were negative for the enzyme at the beginning in culture, almost 60% of 12.5 dpc PGCs showed strong staining after 4-5 hours and 40% after 16-18 hours in culture (Fig. 3, Table 1). However, after the same culture times 16.5-17.5 dpc germ cells were still tTGase negative (not shown).

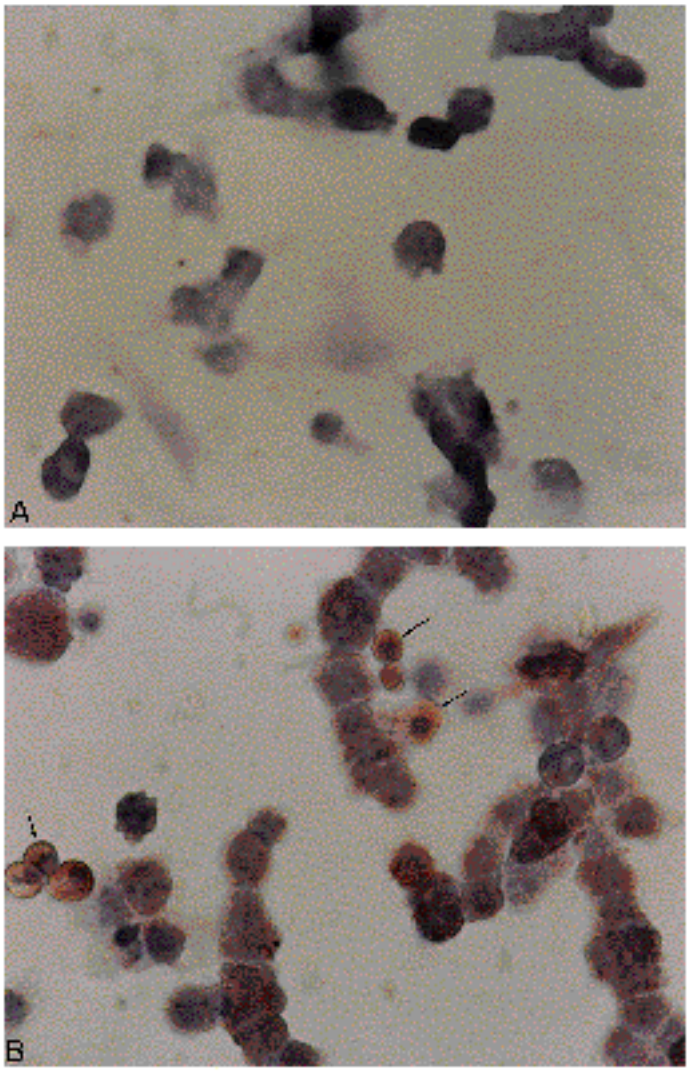

Fig. 3. Immunostaining for tTGase in 12.5 dpc PGCs after 0 hours (A) and 4-5 hours in culture (B). No tTGase-positive cells are seen in A while most of the PGCs are strongly labeled in B; some contaminating hemopoietic cells are also strongly tTGase positive (arrows).

Double staining for alkaline phosphatase (a marker for PGCs) and tTGase performed in cryostat sections of the genital ridges of 12.5-13.5 dpc embryos showed some tTGase-positive PGCs mainly located in extragonadal sites (Fig. 4).

DNA gel electrophoresis

Analysis of total DNA extracted from PGCs cultured for 4-5 hours by 3′-end labeling followed by autoradiography (Fig. 5, line C), indicated internucleosomal cleavage of DNA typical of apoptosis (see, below).

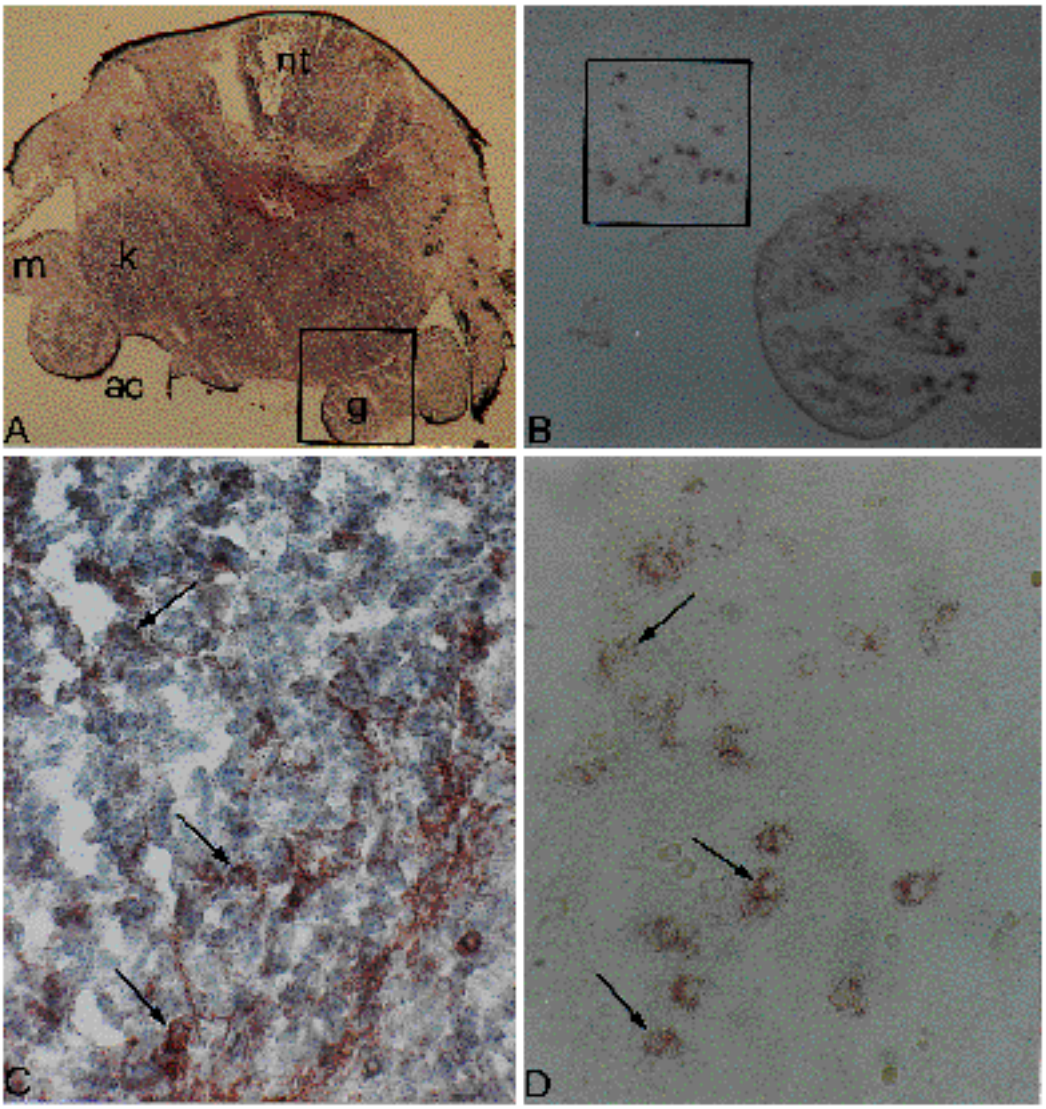

Fig. 4. Localization of PGCs in a 13.5 dpc mouse embryo. (A) Transverse section of the dorsal wall of the embryo through the urogenital region stained with toluidin blue. (B) Magnification of the region shown in the square in A, stained with alkaline phosphatase labeling; PGCs along the splanchnic mesoderm of the wall of the abdominal cavity and within the gonad were identified. (C, D) Magnification of the region shown in the square in B stained with tTGase immunostaining (C) and alkaline phosphatase labeling (D), in two sequential sections; arrows indicate some PGCs positive for both staining. ac, abdominal cavity; g, gonad; k, kidney; nt, neural tube; m, mesonephros.

The effect of cycloheximide, LIF or SCF on PGC apoptosis

One of the most striking features of apoptosis is that, in many systems, it is dependent on protein synthesis (for a review, see Arends and Wyllie, 1991). We found that in the continuous presence of 1 μg/ml cycloheximide the number of PGCs showing apoptotic morphology was markedly reduced (not shown). In addition, the cycloheximide-dependent inhibition of apoptosis was associated with a reduced number of cells showing a positive reaction to the tTGase antibody (Table 1); thus confirming that the enzyme is neosynthesized during the onset of the programmed cell death.

The observation that LIF and SCF are able to increase PGC survival and/or proliferation in vitro (De Felici and Dolci, 1991; Dolci et al., 1991, 1993; Matsui et al., 1991; Godin et al., 1991), prompted us to verify the effect of these compounds on PGC apoptosis. Indeed, the addition of 100 ng/ml SCF or 20 ng/ml LIF to the culture medium prevented the development of tTGase in PGCs after 4-5 hours in culture; this effect was, however, lost after 16-18 hours in culture (Table 1). In addition we found that both growth factors markedly reduced internucleosomal DNA fragmentation occurring in cultured PGCs (Fig. 5).

Table 1. Effect of 100 ng/ml SCF, 20 ng/ml LIF and 1 μM cycloheximide on the number of PGCs positive to polyclonal antibodies against tTGase

	% of tTG-positive PGCs	
Treatment	4-6 hours	16-18 hours
None	57±9	40±6
SCF	12±4	39±2
LIF	22±5	40±8
Cycloheximide	26±1	28±3

Values represent the average of at least three experiments ±s.e.m. Differences between control and treatments, analysed by χ^2 test, were highly significant ($P<0.01$) for cells cultured for 4-6 hours and not significant for cells cultured for 16-18 hours. At the beginning of the culture period no tTGase-positive PGCs were detectable.

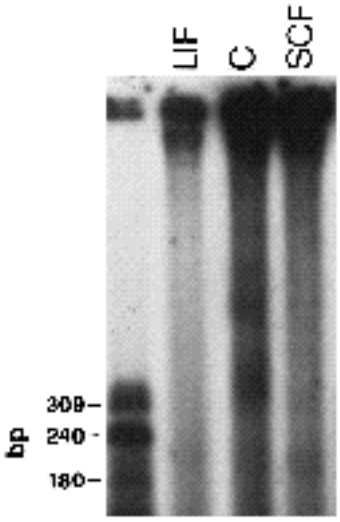

Fig. 5. Electrophoretic analysis of DNA obtained from mouse 12.5 dpc PGCs cultured for 4-5 hours. DNA from control PGCs (lane C) shows a ladder of DNA degradation characteristic of apoptosis.The fragmentation of DNA was markedly reduced in the culture to which 100 ng/ml SCF or 20 ng/ml LIF was added at the start of incubation. The lane on the left shows molecular size markers.

DISCUSSION

Early in vertebrate development primordial germ cells migrate from the hindgut to the genital ridges where, after active mitotic proliferation, they ultimately differentiate into gametes. A consistent observation by us and by others (De Felici and McLaren, 1983; Donovan et al., 1986) is that proliferating PGCs obtained from 11.5-12.5 dpc mouse embryos rapidly die when cultured in vitro in the absence of somatic cell support. Here we show that the death of purified PGCs in vitro occurs with the morphological and biochemical hallmarks of programmed cell death, or apoptosis. Figs 1 and 2 show the morphological hallmarks of apoptosis in 12.5 dpc PGCs after 4-5 hours in culture. At this time, nearly 60% of PGCs were positively labeled with antibodies against tTGase (Fig. 3; Table 1). In addition, the electrophoretic analysis of DNA of PGCs cultured for 4-5 hours showed degradation to oligonucleosomal fragments typical of apoptosis (Shi et al., 1989; Wyllie et al., 1984; Smith et al., 1989; Fig. 5). Lastly, the finding that in the presence of 1 μM cycloheximide, the percentage of PGCs showing apoptotic morphology and positivity to tTGase decreased by about 40% both after 4-5 hours and 16-18 hours in culture (Table 1), provides further evidence for apoptosis. Post-mitotic germ cells obtained from 16.5-17.5 dpc fetal gonads, that we know able to survive for some days in culture (De Felici and McLaren, 1983), neither showed apoptotic phenotype nor tTGase positivity (data not shown).

Since several groups have recently proved the importance of SCF and LIF for the survival and/or proliferation of mouse PGCs in vitro (De Felici and Dolci, 1991; Dolci et al., 1991, 1993; Matsui et al., 1991; Godin et al., 1991), we analyzed the effect of these cytokines on PGC apoptosis. Both factors appeared able to suppress apoptosis in PGCs as shown by their ability to almost completely prevent the expression of tTGase (Table 1) and DNA fragmentation (Fig. 5) after 4-5 hours in culture. However, they appeared unable to inhibit apoptosis over longer culture times since the percentage of PGCs showing tTGase positivity after overnight culture in the continuous presence of cytokines was similar to the control (Table 1). This agrees with our previous observations showing that SCF and/or LIF caused only a small increase in the number of purified 11.5 dpc PGCs over the control after 1 day in culture (Dolci et al., 1993). It is possible that the ability of the soluble forms of SCF and LIF to prevent apoptosis is limited compared with the membrane-bound forms (Dolci et al., 1991) and/or that additional cell-bound signals, as well as soluble one, are necessary to support PGC survival.

Although the notion that cells in higher animals require signals from neighboring cells to avoid suicide by apoptosis is not new, our data show for the first time that such a mechanism might operate in early gametogenesis and provide direct evidence that, in vitro, SCF and LIF act directly on PGCs by suppressing apoptosis. The advantages of having PGC survival depending on signals produced by the surrounding somatic cells may be, for example, to provide a simple mechanism for eliminating cells that end up in abnormal locations during migration. In line with this hypothesis, we reported here that some PGCs positive to tTGase were detectable in extragonadal sites of 12.5 dpc embryos (Fig. 4), thus providing strong support to the notion that apoptosis may have a physiological role in the establishment of germ cell population in the early embryo. As a matter of fact, an active process of cell death may provide a further means of precisely regulating germ cell number by signals that can stimulate or inhibit apoptosis. Since in vivo, the phagocytosis and digestion of apoptotic cells occur rapidly (Bursh et al., 1990), it is not surprising that often the process may be remarkably incospicuous histologically.

The effect of SCF on PGC apoptosis reported here suggests that in the *White spotting* and *Steel* mutations the affected stem cells degenerate by programmed cell death. Lastly, it is tempting to speculate that aberrant survival of proliferating germ cells resulting from apoptosis failure, prolonging the life span of some cells, might lead to development of teratoma and teratocarcinoma stem cells, which have been shown can arise spontaneously or experimentally derivated from PGCs (Stevens, 1967; Matsui et al., 1992; Resnick et al., 1992).

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