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Secretion of stem cell factor and granulocyte–macrophage colony-stimulating factor by mouse embryos in culture: influence of group culture

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Summary

Previous studies showed that the addition of a growth factor to the culture medium could modulate embryo development. The possible secretion of different factors to the culture medium by the embryo itself, however, has been poorly evaluated. The present study was designed to investigate: (1) the influence of single or group culture on the development of 2-cell mouse embryos (strain CD-1) to the blastocyst stage; (2) the release of granulocyte–macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) into the culture medium by the embryo; and (3) the levels of GM-CSF and SCF in the culture medium from both single and group embryos. Two-cell CD-1 mouse embryos were cultured for 96 h singly or in groups of five embryos per drop. GM-CSF and SCF were assayed by ELISA in the complete culture medium. It was found that embryos cultured in groups gave a higher percentage of total blastocyst formation and hatched blastocyst when compared with single embryo culture. The mouse embryos secreted GM-CSF and SCF to the culture medium. The concentration of these cytokines is significantly higher in the group cultures than the level found in single cultures. In conclusion, mouse embryos in culture secrete GM-CSF and SCF to the culture medium and the concentration of these cytokines increases during communal culture. These factors may be operating in both autocrine and paracrine pathways to modulate embryo development during *in vitro* culture.

Keywords: Blastocyst, Embryo, GM-CSF, Group culture, SCF

Introduction

Production of embryos up to the blastocyst stage is now possible in numerous species, although culture conditions do not replace all the benefits of embryo development within the female reproductive tract (Bavister, 2000). Preimplantation development of *in vitro* cultured mouse embryos is slower than *in vivo* cultured (Harlow & Quinn, 1982). In addition, the level of apoptosis in the mouse blastocyst is three-fold higher in *in vitro* cultures than *in vivo* (Brison & Schultz, 1997). An important focus of the research of *in vitro* reproduction is the design of an *in vitro* culture system that resembles *in vivo* development more closely, in order to support better growth of embryos to the blastocyst stage, which then leads to successful implantation. Studies in rodents and other species suggest that cytokines and growth factors regulate blastocyst formation and the rate of development of preimplantation embryos (Hardy & Spanos, 2002). In this regard, it is known that a wide range of growth factors and their receptors is expressed during preimplantation development and that the addition of exogenous growth factors seems mainly to affect blastocyst formation (Hardy & Spanos, 2002).

Previous studies have shown that *in vitro* mouse embryo development can be improved by a reduction

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in the volume of the culture medium (Gardner *et al.*, 1997), as well as by growing communal embryos (Almagor *et al.*, 1996). It has been suggested that this improvement could be caused by a critical concentration of specific embryo-derived factors (Paria & Dey, 1990). A reduction in the incubation volume or an increase in the number of embryos in the culture medium could facilitate reaching the proposed critical concentrations of the specific embryo-derived factors. This fact indicates that embryo-producing growth factors could be operating in an autocrine and/or paracrine way to support embryo development as well as to promote improved pregnancy rates (Paria & Dey, 1990; O'Neill, 1998).

The addition of several factors to the embryo culture medium can modulate *in vitro* embryo development. Among these factors, one can include colony-stimulating factor, leukemia inhibitor factor, transforming growth factor, epidermal growth factor and insulin-like growth factor (Kane *et al.*, 1997). Even though most studies have evaluated the effect of culture medium supplementation with different growth factors, very little is known about the production and liberation of growth factors to the culture medium by the embryo itself under *in vitro* incubation (O'Neill, 1997).

It is known that the cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) is secreted by epithelial cells in the mouse oviduct and uterus (Robertson *et al.*, 1992). Studies performed with a murine model have suggested that GM-CSF can act as a survival factor for the developing embryo (Robertson *et al.*, 2001). In this regard, GM-CSF genetically deficient mice have a retarded blastocyst formation with a diminution of the inner mass size (Robertson *et al.*, 1999).

Conversely, preimplantation embryos express the receptor for stem cell factor (SCF) and addition of SCF to the culture medium significantly enhanced embryo development to late blastocyst and hatching stage (Taniguchi *et al.*, 2004). Previous work demonstrated the presence of SCF mRNA in human (Sharkey *et al.*, 1995) and mouse embryos (Mitsunari *et al.*, 1999), but there is no information regarding the secretion of this factor by the embryo into culture medium.

In this present study, the secretion into culture medium of two cytokines: GM-CSF and SCF by embryos under *in vitro* incubation conditions was evaluated. Determinations were carried out for both single and group cultures of mouse embryos (strain CD-1).

Materials and methods

Animals

Female mice (strain CD-1) 6–8 weeks of age (17–20 g) who were proven breeder adult males were obtained from 'Bioterio del Instituto Nacional de Higiene'. Female mice were housed, five per cage, at constant humidity, temperature and light schedule (12L: 12D). Commercial food and water were provided *ad libitum.* This protocol was previously approved by the Institutional Review Board.

Collection of embryos

All experiments were performed in accordance with the *Guide for Care and Use of Laboratory Animals* (National Academy of Sciences, 1996). Female mice were given a single i.p. superovulatory doses of pregnant mare serum gonadotropin (5 IU PMSG, Sigma) followed 48 h later by doses of HCG (5 IU, Sigma). The animals were then mated with proven breeder adult males. The copulation plug was checked 24 hours later. The mice were killed by cervical dislocation 46–48 hours after HCG administration. Two-cell-stage embryos were flushed out from fallopian tubes with gamete medium (VitroLife) and washed thoroughly four times before incubation in the culture medium.

Culture conditions

The embryos were cultured for up to 96 h in drops of 20 μl of IVF medium (VitroLife), singly ($n = 19$) or in groups of five embryos per drop (22 drops, $n = 110$), under mineral oil (Sigma) in an atmosphere of 5% CO_2/95% air at 37 °C. After 96 h of culture, the embryos were evaluated. The developmental stage of embryos in culture was assessed visually with the aid of a phase contrast microscope. The embryos were classified as follows: (i) embryos that reached the blastocyst stage (non-hatched plus hatched) and (ii) embryos that remained at the 2-cell or morula stage (cleavage arrest). Non-hatched blastocysts were identified as those that kept inside the zona pellucida. Hatched blastocysts were those completely free of the zona pellucida.

ELISA quantification of SCF or GM-CSF in the embryos drop culture medium

After 48 h of embryo culture, total drops of culture medium, from embryos either cultured singly or in groups, were recovered. The medium was immediately frozen and kept at −70 °C until analysis for GM-CSF and SCF. Murine GM-CSF and SCF in a sandwich ELISA development kit (Peprotech EC) was used to quantify the concentration of these growth factors in the culture medium.

Statistical analysis

Statistical analysis was performed by chi-squared test and Student's *t*-test, using statistic software. Values of $p < 0.05$ were considered statistically significant.

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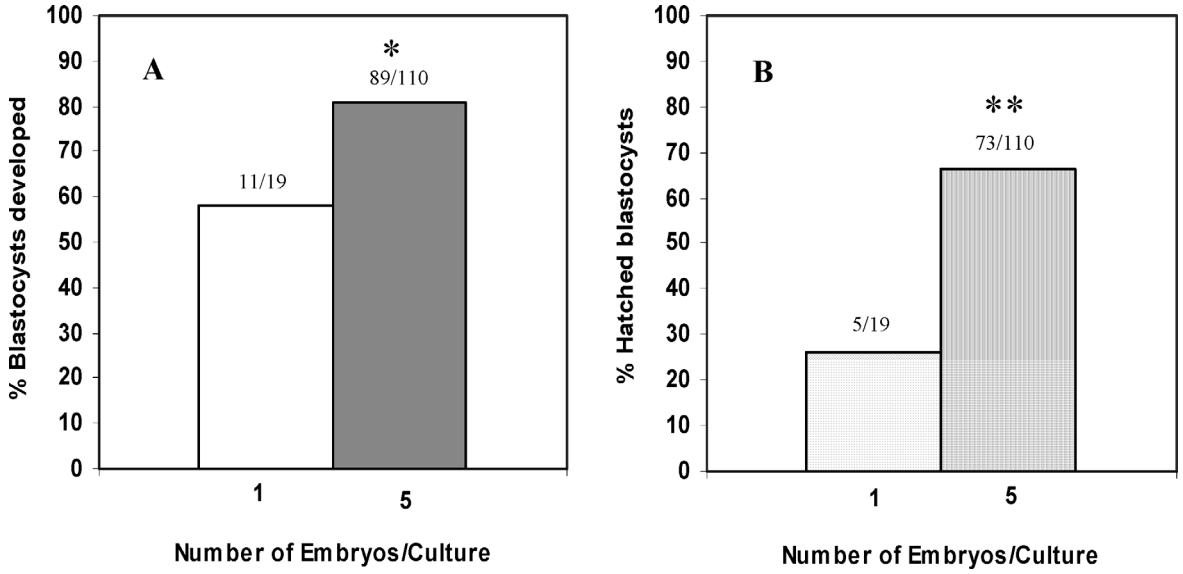

Figure 1 Development of blastocysts following culture of 2-cell embryos singly or in groups of five embryos in 20 μl of medium for 72 h. (*a*) Total number of blastocyst as a function of the number of embryos cultured. Numbers on each bar indicate the number of blastocysts developed/the number of embryos cultured. (*b*) Percentage of hatched blastocysts following culture of 2-cell embryos singly or in groups of five embryos in 20 μl of medium for 72 h. Numbers on each bar indicate the number of hatched blastocysts/the number of embryos cultured. Data were compared by chi-squared analysis, $*p < 0.04$; $**p < 0.03$.

Results

The effect on embryo development of single or group embryo culture

As shown in Fig. 1*a* only 57.9% of the embryos cultured singly were able to reach the blastocyst stage ($p < 0.004$), while 80.9% of the embryos cultured in groups reached the blastocyst stage ($p < 0.03$). When the total embryos that reached the blastocyst stage were further analysed, it was found that 26.3% of singly cultured embryos were hatched blastocyst while 66.4% of the group-cultured embryos were hatched blastocyst (Fig. 1*b*).

GM-CSF and SCF assay in the embryo culture medium

Table 1 shows that GM-CSF and SCF were detected in the embryo culture medium. The concentration of GM-CSF from the group culture condition was twice (8566 ± 840 ng/ml) that found under single culture conditions (4562 ± 882 ng/ml) ($p < 0.02$). Conversely, the concentration of SCF was five-fold higher in the medium from embryos cultured in groups (213.5 ± 28.4 ng/ml) when compared with single cultured embryos (39.9 ± 6.2 ng/ml) ($p < 0.001$).

Table 1 Concentration of GM-CSF and SCF in medium following culture of 2-cell embryos singly or in groups of five embryos in 20 μl of medium for 48 hours.

	Embryos cultured singly	Embryos cultured in groups	*p* value
GM-CSF (ng/ml)	4562 ± 882	8566 ± 840	<0.02
SCF (ng/ml)	39.9 ± 6.2	213.5 ± 28.4	<0.001

GM-CSF and SCF were not detectable in the culture medium at the onset of the embryo culture. $n = 8$ drops per condition. Data are expressed as means ± E.S. The *p* value was obtained using Student's *t*-test.

Discussion

The preimplantation embryo develops for several days within the female reproductive tract (five in mice) in the absence of direct cell contact with this tract, without blood supply and dependent upon luminal secretions from the oviduct and the uterus for its nutrition. Its cell division, apoptosis and differentiation are influenced by the environment, including growth factors (Hardy & Spanos, 2002). Embryos cultured *in vitro*, therefore, grow in the absence of the paracrine influence from oviductal- and uterine-derived factors. A number of growth factors have been showed to affect the rate of embryo development, the proportion of embryos reaching the blastocyst stage, the blastocyst cell number, their metabolism and apoptosis (Hardy & Spanos, 2002). Autocrine secretion of growth factors by embryos and expression of specific receptors at particular cell stages clearly indicate that growth factors play an important role in the early development

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of preimplantation embryos (O'Neill, 1998; Kane *et al.*, 1997).

Deficiencies of growth factors during *in vitro* embryo culture could be supplied either by the addition of these factors to the culture medium or by the co-culture of embryos on monolayers of epithelial cells (from ovarian follicle, oviduct, endometrium or Vero cells) (Freeman *et al.*, 1993). Co-cultures, however, are labour-intensive to maintain in a unit for *in vitro* fertilization and there is an important possibility of cross-contamination (Freeman *et al.*, 1993). Conversely, the supplementation of the culture medium with exogenous growth factors has been show to be beneficial for preimplantational development (Hardy & Spanos, 2002), but their use could not be widely applied as a part of the culture medium until evaluation of the possible consequences on the individual's development, including the adult period after birth. It has been previously suggested that the embryos themselves may be producing growth factors that could act in autocrine and paracrine pathways (Hardy & Spanos, 2002), which is in agreement with our results.

Our data showed that group-cultured embryos presented a higher percentage of formation of total blastocysts and hatched blastocysts when compared with single culture (Fig. 1). This finding is in agreement with previous reports that showed that only 49% of single cultured embryos developed to blastocyst, while 80% of those cultured in groups developed to this stage (Paria & Dey, 1990; Lane & Gardner, 1992; O'Neill, 1998).

It is important to mention that our study provides, for the first time, experimental evidence that the embryo can secrete SCF and GM-CSF (Table 1). The concentration of these growth factors clearly is influenced by the presence of several embryos in the culture medium. There are several reports showing that addition of GM-CSF to embryos cultured *in vitro* is beneficial for their development: (1) GM-CSF enhances the viability of porcine embryos under defined culture conditions (Cui *et al.*, 2004) and promotes human blastocyst development *in vitro*, hatching and the increase in size of the inner cell mass (Sjoblom *et al.*, 1999); (2) the supplementation of the embryo culture medium with GM-CSF decreases the number of cells staining for apoptosis and the expression of antiapoptotic Bcl-2 (Behr *et al.*, 2005); (3) the incubation of blastocysts with recombinant GM-CSF promotes glucose uptake and enhances proliferation and/or viability of blastomeres in murine preimplantation embryos (Robertson *et al.*, 2001); (4) the addition of GM-CSF to the embryo culture medium increases the proportion of transferred embryos that generate a viable progeny and alleviates the effects of *in vitro* culture on fetal and postnatal growth trajectory (Sjoblom *et al.*, 2005); and (5) mouse embryos express the GM-CSF receptor (Robertson *et al.*, 2001).

Our study also shows that the embryo can secrete SCF to the culture medium. The effect of SCF on preimplantational embryos has not been well studied, although embryos grown in media containing SCF have better blastulation and hatching (Taniguchi *et al.*, 2004). SCF mRNA was expressed in the spreading blastocyst as well as the c-Kit protein (SCF receptor) in mice and human embryos (Sharkey *et al.*, 1995; Mitsunari *et al.*, 1999). In addition, others studies have reported that embryos treated with hydrogen peroxide and grown in the presence of SCF have better morphology, faster development, better blastocyst morphology and a lower number of apoptotic cells than those growing without SCF (Kurzawa *et al.*, 2004). It is clear that SCF is beneficial for preimplantation development. Apparently, SCF could be acting to prevent the consequences of oxidative stress *in vitro*, apoptosis and cell degeneration (Kurzawa *et al.*, 2004).

It is important to point out that there are other growth factors that can modulate the embryo development (Kane *et al.*, 1997) and that could be acting in synergy or in an additive way for a better embryo development.

To the best of our knowledge, our results provide, for the first time, experimental evidence that mouse embryos in culture can secrete GM-CSF and SCF and the concentrations of these factors are increased under group culture conditions. These factors may be operating in an autocrine and paracrine way to modulate embryo development during *in vitro* cultures.

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