Protective potential of SCF for mice preimplantation embryos cultured *in vitro* in suboptimal conditions

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Received: 5 May 2008 / Accepted: 22 August 2008 / Published online: 17 September 2008
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Abstract

Purpose To examine the effect of stem cell factor (SCF) to embryos exposed to detrimental factors.

Methods Mice embryos cultured in control medium or Exp.1. with FasL or FasL+SCF Exp.2. with hydrogen peroxide (HP) or HP+SCF; Exp.3. frozen–thawed and cultured with or without SCF. Immunohistochemistry for Fas and c-kit receptors was performed in blastocysts. Blastocyst rates, total numbers of blastocyst cells (TB) and inner cell mass cell counts (ICM) were determined.

Results Immunohistochemical studies revealed expression of both Fas and c-kit in blastocyst cells. Exp.1. Significantly more blastocysts were found in control when compared to FasL group and to FasL+SCF group. TB and ICM counts in control and FasL+SCF group were significantly higher comparing to FasL group. Exp.2. We found significant differences between three groups in all three evaluated parameters. The highest blastocyst rates, TB and ICM counts were found in control, lower in HP+SCF group and the worst in HP group. Exp.3. No significant differences in TB and ICM

counts were found. More embryos formed blastocyst in control than in two cryopreserved groups. Blastocyst rates did not differ between two cryopreserved groups.

Conclusion SCF may improve culture of embryos exposed to unfavorable milieu.

Keywords Embryo culture · FasL · Hydrogen peroxide · SCF

Introduction

Human embryo culture to blastocyst is still relatively inefficient. The *in vitro* conditions differ from *in vivo* environment of the female reproductive tract where the mechanisms protecting embryos from the impact of detrimental factors exist [1, 2]. The embryos developing *in vitro* may be exposed to many biological as well as physical factors, and they grow in a milieu deprived from survival and growth promoting factors necessary for optimal embryo development.

Such factors as reactive oxygen species (ROS), Fas ligand (FasL) or freezing–thawing procedure have detrimental impacts on preimplantation embryo development that may have significance also in clinical medicine, because the embryos developed in *in vitro* fertilization programs may be exposed to all these factors. Detrimental impact of ROS [3–5], and cryopreservation [6–8] on preimplantation embryos is well documented. On the other hand, relatively poor and preliminary information regarding the influence of Fas/FasL system on preimplantation embryo development exists [9, 10]. Fas receptor is a member of TNF/NGF receptor family inducing apoptosis. The native agonist for Fas is Fas ligand protein that is similar to tumor necrosis factor. In target cells that express

SCF—Stem cell factor has protective potential for mice preimplantation embryos exposed to Fas ligand or hydrogen peroxide but not for embryos that underwent freezing–thawing procedure.

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Fas [11–13] interaction FasL/Fas leads to activation of caspase 8 via FADD/MORT1 “death protein.” Activated caspase 8 activates other caspases causing apoptotic cell death [14, 15]. The FasL-induced cell death *in vitro* in several cell types expressing Fas has been reported previously [16–18].

The problem of prevention of embryo cells malfunction or even death in *in vitro* conditions is of great interest. Many factors were found to improve the preimplantation embryo development so far, but the optimal composition of embryo culture media has not been established yet.

SCF (Stem cell factor, Kit ligand) is a proven survival factor for several intensively proliferating cell types including primordial germ cells (PGC) [19, 20], hematopoietic stem and progenitor cells [21–23]. Unfortunately, little is known on its effects on embryos, although several other survival factors for early embryos have been studied so far [24–27]. Therefore, the purpose of the study was to examine the effects of SCF in embryos exposed to growth inhibiting factors.

Materials and methods

Female B6C3F1 mice (6–8 weeks old) were treated intraperitoneally with 5 IU of pregnant mares’ serum gonadotropin (Folligon, Intervet, Belgium), followed 48 h later by an injection of 10 IU of hCG (Chorulon, Intervet, Belgium) and mating with DBA males. The copulation plug was checked after 24 h. The mice were killed 40 h after hCG injection by cervical dislocation and the two cell embryos were flushed out from the fallopian tubes. The embryos were incubated in control medium *i.e.* Earl’s balanced salt solution (EBSS, Sigma, USA) supplemented with sodium pyruvate (0.33 mM), sodium lactate (21.4 mM) and human serum albumin (4 g/L; HSA, Sigma, USA). After an initial 6 h of incubation the embryos which passed the two cell block were introduced to the experiments.

Based on the results of our previous paper [28], for all experiments described below the concentration 100 ng/mL of SCF was chosen as the most effective.

Experimental design

Experiment 1. The embryos were randomly transferred to control medium—group 1A, medium containing 0.5 µg/mL FasL (Sigma, USA)—group 1B, or medium with 0.5 µg/mL FasL and 100 ng/mL of SCF (Sigma, USA)—group 1C. The concentration of FasL was established in a separate set of experiments (data not included) as the concentration which inhibited embryo

growth, allowing formation of only very few blastocysts.

Experiment 2. The embryos were randomly transferred to control medium—group 2A, medium with 0.1 mM hydrogen peroxide—group 2B, or medium with 0.1 mM hydrogen peroxide and 100 ng/mL of SCF—group 2C. The concentration of hydrogen peroxide which impaired embryo growth was set based on the results published previously [29].

Experiment 3. The embryos were transferred to control medium—group 3A, frozen–thawed and cultured thereafter in control medium—group 3B, or frozen–thawed and cultured in presence of 100 ng/mL of SCF—group 3C. The commercially available media with propanediol and sucrose (Embryo Freezing Media, Embryo Thawing Media, Medicult) were used for cryopreservation and thawing. The embryos were cryopreserved in CBS straws (Cryo Bio Systems, France) with freezing controller (type CL-863, Cryo-logic, Australia). A multi-step computer controlled freezing protocol corresponding to freezing media user’s manual was used. Thawing was carried out according to thawing media user’s manual.

All embryos were cultured for 96 h in groups of ten embryos in 50 µL droplets under mineral oil (Sigma, USA) in Petri’s dishes (Corning, USA) at 37°C and 5%CO₂/5%O₂.

The experiments were approved by the local Ethical Commission.

Outcome measures In each experiment the blastocyst rates, total counts of blastocyst cells (TB) and inner cell mass cell counts (ICM) following 96 h of culture in control and experimental embryos were determined. Additionally, in one series of experiments Fas and SCF receptors were identified in the embryos.

Blastocyst rates (percentages of embryos reaching the blastocyst stage) were checked in inverted phase-contrast microscope (Eclipse 2000, Nikon, Japan).

Total blastocyst and inner cell mass cell counts (TB and ICM respectively) were identified using a modified De la Fuente and King method for differential staining [26, 30]. The *zona pellucida* was damaged by exposure for 5 to 10 min to 0.1% pronase (Sigma, USA) at 37°C. Subsequently, the embryos were transferred for 10 to 15 min to PBS supplemented with 10 µg/ml of calcium ionophore A23187 (Sigma, USA), 10 µg/ml of propidium iodide (Sigma, USA) and 1 µg/ml of Hoechst 33342 (Sigma, USA). The specimen was evaluated under fluorescent

microscope (Axioscop, Zeiss, Germany) using 345 nm ultraviolet light filter. The cells were counted using image analysis software (Microimage, Olympus, Japan).

Identification of Fas and c-kit receptors was performed to determine whether mice embryos are capable to respond to SCF and (or) FasL stimulation. The immunocytochemical reactions were carried out on deparaffinized and rehydrated sections (4 μ m). The endogenous peroxidase was inhibited with 3% H₂O₂ in PBS. The nonspecific binding sites were blocked with 2.5% normal horse serum (Sigma, USA) for 30 min. The sections were incubated overnight at 4°C with primary antibody: anti-mouse Fas/TNFRSF6 antibody (goat polyclonal, R&D Systems, UK) and anti-c-kit (goat polyclonal, Santa Cruz Biotechnology, USA). Subsequently sections were treated with biotinylated secondary antibody, and streptavidin–horseradish peroxidase complex (Vectastain Kit, Vector Laboratories, USA). 3,3'-diaminobenzidine (DAB) was used to visualize the immunohistochemical reaction. The sections were counterstained with Mayer's haematoxylin. In the control reaction, the primary antibody was replaced by normal goat serum. Finally, sections were examined with light microscope.

Statistical analyses

In experiments 1, 2 and 3, respectively 16, 18 and 16 independent experiments were performed. Data are presented as mean $\pm$ SD. The data were analyzed by a non-parametric analysis of variance (ANOVA—multiple comparison test) for more than two variables. The calculations were done using Statistica for Windows (StatSoft, Inc., Tulsa, OK., USA). Statistical significance was accepted at $p < 0.05$.

Results

Immunohistochemical studies revealed expression of both Fas and c-kit receptors in trophoectoderm as well as in inner cell mass of mice blastocysts (Fig. 1). In both reactions there was no immunostaining when primary antibodies were omitted.

Experiment 1. In the control group (1A) significantly more blastocysts after 96 h of culture were found (82.3% $\pm$ 28.9) when compared to FasL (2B) (54% $\pm$ 34.7) and FasL+SCF group (1C) (68.8% $\pm$ 19.5). The blastocyst rate in FasL+SCF group (1C) did not differ from FasL group (1B). We found markedly reduced TB (49.8 $\pm$ 7.4) as well as ICM (13.1 $\pm$ 2.9) counts in embryos exposed to FasL (1B) when compared to

Experiment 2.

control embryos (group 1A. TB—59.6 $\pm$ 12.2; ICM—17.3 $\pm$ 5.1) and to embryos cultured with FasL and SCF (group 1C. TB—54.9 $\pm$ 5.6; ICM—15.1 $\pm$ 2.3) (Fig. 2). SCF at the concentration of 100 ng/ml had a positive effect on embryos cultured with 0.1 mM hydrogen peroxide. We found significant differences between three groups in all three evaluated parameters. The highest blastocyst rate, TB and ICM counts (respectively 82.3% $\pm$ 28.9; 59.6 $\pm$ 12.2; 17.3 $\pm$ 5.1) were found in control group (2A), lower in group 2C—embryos cultured with hydrogen peroxide and SCF (51.6% $\pm$ 23.4; 42.5 $\pm$ 7.5; 10.14 $\pm$ 3.0) and the worst in group 2B exposed to hydrogen peroxide only (27.8% $\pm$ 25.8; 31.8 $\pm$ 12.9; 6.0 $\pm$ 4.9) (Fig. 3).

Experiment 3.

In control group (3A) significantly more blastocysts were found (82.3% $\pm$ 28.9) comparing to cryopreserved embryos (group 3B. 50.8% $\pm$ 22.5) as well as to embryos cultured with SCF following freezing–thawing procedure (group 3C. 67.1% $\pm$ 15.3). The rates of blastocyst between the two latter groups did not differ significantly. The analysis of TB (3A—59.6 $\pm$ 12.2; 3B—56.4 $\pm$ 5.4; 3C—58.1 $\pm$ 4.3) and ICM (3A—17.3 $\pm$ 5.1; 3B—15.8 $\pm$ 2.2; 3C—16.5 $\pm$ 1.8) counts did not reveal any significant differences between the groups (Fig. 4).

Discussion

It has been well documented that the preimplantation embryo requires specific growth factors for proper development. Tubal mucosa and endometrium are sources of cytokines and growth factors which are necessary for embryos [31–33]. It is obvious the embryos cultured *in vitro* are deprived from majority of these factors.

The culture of mice embryos to blastocyst stage does not require the use of sequential culture systems, (in contrast to human IVF) and the majority of mice embryos reach blastocyst in non-sequential IVF media [34]. We had approximately 80% of blastocysts in control medium, which did not differ from that reported in other papers [24, 35, 36]. Our idea was to avoid overlapping of investigated conditions or factors and numerous agents present in complex sequential culture systems (i.e. IGFs, insulin, energy sources).

Fig. 1 Immunohistochemical reactions. Immunostaining for c-kit (a) and for Fas (c) was visualized in both trophoectoderm and inner cells mass cells. No immunostaining was found in negative control for c-kit (b) and for Fas (d). In negative control reactions the primary antibodies were replaced with normal serum

There are significant differences between mice strains in tolerance of *in vitro* conditions. Additionally it has been widely reported that zygotes (2PN) retrieved from oviducts develop poorer than two-cell embryos. The goal of our study was to examine the significance of SCF during the time of expected expression of c-kit in embryos which passed the two cell block. Such an approach is widely used and was reported elsewhere [10, 36, 37].

It is well known that the majority of good quality embryos develop to blastocyst on day 5 following fertilization. On day 5 or 6 (day 3 or 4 of culture) they are partially or completely hatched. On the other hand there are embryos developing slightly slower which form blastocysts on day 6. We extended the experimental follow-up to day 6 to avoid the bias caused by underscoring

of embryos of the latter group, which can produce quite good quality blastocysts somewhat later. Such an experimental design was also used previously [36, 38]. Needless to say, in human IVF programs the transfer of blastocyst on day 6 following fertilization is also used.

The mRNA for c-kit has been found to be expressed within mouse embryos from two cell to blastocyst [39]. In human, both the c-kit and SCF mRNAs have been detected in preimplantation embryos, suggesting its autocrine activity [40]. Immunoeexpression of c-kit in mouse blastocysts strongly suggests SCF's role in regulation of early embryo development. It was previously reported that SCF–c-kit interaction enhances *in vitro* preimplantation embryo development as well as embryonic stem cells differentiation [41, 42]. In our previous study we found no beneficial effect of

Fig. 2 Blastocyst rates, TB and ICM cell counts (mean $\pm$ SD) following culture in control medium, in exposure to FasL and in exposure to FasL and SCF. *A*: $p<0.05$ vs. Control, *B*: $p<0.05$ vs. FasL; ($n=16$ independent experiments)

SCF on blastocyst rate, TB and ICM counts of embryos cultured in optimal culture conditions (without any inhibiting or detrimental agents). However, we have found significant positive effect of SCF on development of embryos exposed to TNF alpha [28]. In the current research we continued to assess the influence of SCF on preimplantation embryos growing in unfavorable conditions generated in other experimental models.

As it was mentioned in the introduction Fas ligand (Fas-L) is a proven apoptosis activator. The simultaneous expression of Fas mRNA as well as Fas-L mRNA (and Fas protein) at two-cell (in rat) and four-cell (in human) preimplantation embryos were previously reported [9]. Also, the human fragmented embryos often express both Fas and Fas-L mRNA [9]. It may suggest the involvement of Fas–Fas-L system in autocrine apoptotic pathways in

early embryos. In the study we determined the expression of Fas receptor in blastocyst cells using immunohistochemical reaction. We have also found that Fas-L impaired the mouse embryo preimplantation development reducing the number of embryos that reached the blastocyst stage. Fas-induced inhibition of two-cell mouse embryos *in vitro* development has been reported previously [10]. Additionally we found significantly reduced cell counts (both TB and ICM) in embryos treated with Fas-L. The most likely reason of this effect was induction of apoptosis by stimulation of Fas receptor.

Keeping in mind Fas–Fas-L interaction, the question may be risen if SCF may prevent (or revert) unfavorable influence of Fas-L on *in vitro* cultured embryos. In our experiments, SCF increased the blastocyst rate of embryos treated with Fas-L but the effect was insignificant.

Fig. 3 Blastocyst rates, TB and ICM cell counts (mean $\pm$ SD) following culture in control medium, in exposure to hydrogen peroxide and in exposure to hydrogen peroxide and SCF. *A*: $p<0.05$ vs. Control, *B*: $p<0.05$ vs. HP; ($n=18$ independent experiments)

Fig. 4 Blastocyst rates, TB and ICM cell counts (mean $\pm$ SD) following culture of fresh embryos in control medium, culture of cryopreserved embryos in control medium, culture of cryopreserved embryos in exposure to SCF. A: $p<0.05$ vs. Control, B: $p<0.05$ vs. Cryo; ($n=16$ independent experiments)

However, we found significant increase of TB as well as ICM cell counts in embryos cultured in medium containing both SCF and Fas-L when compared to those cultured with Fas-L only. Thus, SCF seems to provide the compensative effect for an unfavorable influence of Fas-L on preimplantation embryos *in vitro* development.

In the next experimental model, an attempt was made to determine whether SCF could compensate for the unfavorable influence of oxidative stress created by the addition of hydrogen peroxide (HP)—a donor of reactive oxygen species (ROS). It has been reported that HP may be spontaneously generated in *in vitro* embryo cultures [3–5]. Hydrogen peroxide may damage the cells through apoptosis (either directly or via a second messenger) or induce necrosis [43–45]. The preimplantation embryos are deprived from the protective mechanisms such as activation of catalase, glutathione peroxidase and glutathione. Catalase may be expressed from the blastocyst stage [46], glutathione and its peroxidase are synthesized only by oocytes and by cumulus cells [47]. The protection of preimplantation embryos developing *in vivo* against oxidative stress is realized by hypotaurine secreted by epithelial cells of the fallopian tubes. Therefore the embryos cultured *in vitro* are deprived of this protection. Disruptive effect of reactive oxygen species is important in human and veterinarian medicine. High ROS levels in culture media are responsible for increased embryonic fragmentation, slow cleavage rate and lower pregnancy rates in assisted reproductive techniques cycles [48]. To improve the outcome of the procedure, properties of various antioxidants have been studied [3, 49]. On the other hand, the effect of growth factors has been poorly investigated. We have previously reported favorable effect of IGF1 IGF2, EGF and SCF on embryos exposed to HP [29]. The current

study confirms the previous results and adds on new data, showing positive effects of SCF on TB and ICM counts.

The final part of the study was performed to evaluate the potential rescue effect of SCF on embryos that underwent cryopreservation. It is well documented that the freeze–thaw procedure has certain detrimental impact on post-thaw embryo growth. It may cause destruction of the cell membrane integrity, redistribution of actin fibers, mitochondrial dysfunction as well as increased formation of reactive oxygen species [6, 8]. The effect of growth factors and cytokines on post-thaw embryo development has not been extensively investigated. However, the protective effects of co-culture systems with Vero cells as well as particular factors such as insulin-like growth factor 1 and 2 (IGF1, IGF2), leukemia inhibitory factor (LIF) or epidermal growth factor (EGF) on post thaw growth of mouse embryos were reported previously [50, 51]. In the current study we determined that the freeze–thaw procedure caused significant decrease of blastocyst rates. On the other hand, both TB and ICM cell counts did not differ significantly between control and cryopreserved embryos group. These results are in agreement with current opinion that cleavage stage embryos, which survived freezing–thawing procedure, can produce healthy blastocysts composed of proper number of cells. We found no differences in examined parameters between embryos cultured in control medium after thawing and those cultured with SCF following cryopreservation procedure. Thus SCF revealed no rescue effect toward the cryopreserved embryos. Most likely the freezing–thawing procedure caused irreversible damage and arrested the embryo development.

The beneficial influence of SCF on blastocyst rates or TB and ICM of blastocyst cultured in exposure to apoptosis inducing agents (hydrogen peroxide, Fas-L) compared with

lack of such effect in blastocyst developing in optimal culture conditions may indicate the antiapoptotic activity of this factor. Such an activity the other growth factors have too. IGF1 and IGF2 showed an antiapoptotic effect on the mouse embryo cells, when the apoptosis was induced by TNF α [52]. IGF1 may play a role as apoptotic survival factor in early human embryo [53]. Such a potential of SCF in non-embryonic, however non-differentiated cell populations has also been reported [20, 23]. Particularly interesting is the SCF protection of hematopoietic precursor cells from Fas mediated apoptosis [14, 54]. The outcomes of our study suggest that Fas-L and ROS effects on preimplantation embryos may be partly reversed by addition the SCF to culture media. This is in agreement with our previous report concerning the positive effect of EGF, IGFs and SCF on development of embryos cultured in presence of TNF α that uses very similar pathway to induce the apoptosis in cells as FasL does [28]. Therefore the effect of SCF might be mediated by the same mechanisms as in the hematopoietic precursor cells.

It is postulated that such factors as hydrogen peroxide initiate the cell damage by the destruction of mitochondrial membranes. In contrast, Fas-L similarly to TNF α acts through the receptor mediated activation of the caspases—the family of proteases involved in the execution of cell death [55, 56]. Thus, the protective potential of tested factor is probably caused not only by its antiapoptotic, but also a promitotic activity.

SCF seems not to improve the development of “healthy” embryos growing in optimal culture conditions, but to provide the certain compensative effect for an experimentally designed unfavorable *in vitro* milieu. However, still the question may be raised if the embryos rescued with SCF and other growth factors develop into normal and healthy offspring. This issue still awaits further research.

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