Growth factors effects on preimplantation development of mouse embryos exposed to tumor necrosis factor alpha

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SUMMARY

The success rates of assisted reproduction techniques are still unsatisfactory. Relatively few in vitro cultured embryos reach the blastocyst stage. The purpose of the study was to evaluate the protective potential of epidermal growth factor (EGF), insulin-like growth factors 1 and 2 (IGF-I, IGF-II) and stem cell factor (SCF) on in vitro development of pre-implantation mouse embryos exposed to tumor necrosis factor alpha (TNFα). C3B6F1 female mice were superovulated with 5 IU of pregnant mare serum gonadotropin (PMSG) and 48 h later with 5IU of equine chorionic gonadotropin (eCG). Following the second injection females were mated with DBA males. Two cell embryos were flushed out from the fallopian tubes 40h after eCG administration. After retrieval, the embryos were divided into control and
experimental media and incubated in groups of ten for 96h (37°C, 5%CO₂, in droplets of 50 µl under mineral oil). In the first part of experiment, the embryo development was tested in media containing EGF, IGF-I, IGF-II, SCF, TNF-α (1 to 1000 ng/ml). In the second part of the study, the development of embryos was examined in medium containing 100 ng/ml TNFα and one of following factors: IGF-I, IGF-II; EGF or SCF (100 ng/ml). During the culture embryos were examined at 24 hours intervals to assess the embryo development. Blastocyst rate was determined following 96 hours of culture. Evaluation of total blastocyst cell number (TB) and inner cell mass (ICM) was also performed. TNFα significantly reduced (p<0.05) the blastocyst rates as well as TB and ICM. The examined growth factors improved the development of embryos exposed to TNFα. Thus, in this study, the protective action of IGF-I and II, EGF and SCF against the detrimental influence of TNFα was demonstrated. Reproductive Biology 2005 5(1): 83-99. **Key words:** mouse, embryo; TNFα, IGF-I; IGF-II; EGF; SCF; *in vitro* culture

**INTRODUCTION**

Extended culture of human embryos to blastocyst stage is a procedure of increasing importance in *in vitro* fertilization (IVF) programs. Utilization of this technique is still influenced by high costs of culture media and relatively low rates (30-60%) of embryos reaching the blastocyst stage [12, 14, 19, 29]. The *in vitro* cultured embryos are exposed to many potentially harmful factors and they develop in sub-optimal conditions, which differ greatly from environment of the female reproductive tract [2, 40]. The typical external factors influencing the *in vitro* embryo development include temperature, pH and osmotic pressure. Moreover, medium composition (the presence or absence of energy sources and growth promoting factors) and finally presence of damaging substances such as free oxygen radicals play a role [32]. The *in vivo* conditions within the oviduct and uterine cavity constitute the optimal environment providing not only proper pH, temperature and ion concentrations but also stable sources of the nutrients and growth pro-
moting factors [3, 37]. The composition of the culture medium mimicking the natural environment including interleukins and growth factors may be crucial for the success of the IVF procedures [1, 9, 10, 11, 41, 42, 43]. The influence of epidermal growth factor (EGF) as well as insulin-like growth factors 1 and 2 (IGF-I and IGF-II) on mouse embryo development has been earlier reported by our group and other investigators [16, 18, 24, 27, 28]. It seemed reasonable to compare the effects of these factors in optimal culture conditions vs. culture in exposition to detrimental factor. The protective potential of EGF, IGF-I and IGF-II toward the embryos exposed to the inhibiting influence of oxidative stress was confirmed in our previous study [23]. Anti-apoptotic effect of stem cell factor (SCF) has been previously reported for some poorly differentiated cells but not of preimplantation embryos [13].

The purpose of the study was to investigate the effect of EGF, IGF-I, IGF-II and SCF on in vitro development of preimplantation mouse embryos exposed to tumor necrosis factor alpha (TNFα) - a biological agent capable of inhibiting the preimplantation embryo development.

**MATERIALS AND METHODS**

The study was performed on 6–8 weeks old female mice of C3B6F1 strain and males of the DBA strain. Mice were raised in a 12-h day/night cycle. Female mice were treated intraperitoneally with 5 IU of pregnant mare serum gonadotropin (PMSG; Foligon, Intervet, Belgium). An injection of 5 IU of equine chorionic gonadotropin (eCG; Chorulon, Intervet, Belgium) was given 48 hours later and the animals were mated. The copulation plug was checked 24 hours later. Mice were killed 40 hours after eCG injection by cervical dislocation. Two-cell stage embryos were flushed out from the fallopian tubes. The embryos were subsequently incubated in Earle’s balanced salt solution (EBSS, Sigma, USA) with sodium pyruvate (0.33 mM), sodium lactate (21.4 mM) and 4 g/l human serum albumin (HSA, Sigma, USA) – the medium commonly used in human IVF laboratories. After 6 hours of initial incubation the embryos were divided and transferred to
control medium (without growth factors) or experimental culture medium enriched with different concentrations of one of the tested factors.

All embryos were cultured for 96 hours in groups of 10 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 development of embryos was evaluated in media containing different concentrations (1, 10, 100, 1000 ng/ml) of EGF, IGF-I, IGF-II, SCF, TNFα (Sigma, USA). In the second part of the study, the development of embryos was tested in medium containing TNFα (100 ng/ml) and one of the following factors IGF-I, IGF-II; EGF or SCF (100 ng/ml). The culture conditions were similar to those described above. The concentrations of tested factors were chosen based on first part of the study.

**Evaluation of embryo morphology under the phase-contrast microscope**

In each experiment, the embryos were examined at 24 hours intervals to assess the embryo development. The blastocyst rates (percentage of embryos reaching the blastocyst stage) following 96 hours of culture in control medium and media containing growth factors were statistically evaluated.

**Differential staining**

Differential staining [8, 24] was used to evaluate total number of blastocyst cells (TB) and number of cells in inner cell mass (ICM). Both these parameters correlate well with blastocyst quality. The zona pellucida was removed by exposure for 5 to 10 min to 0.1% pronase (Sigma, USA) at 37°C. Then, 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).
**Blastocyst cells viability evaluation**

The procedure was similar to differential staining. However, the incubation mixture was deprived of the calcium ionophore. Dead cells were stained with propidium iodide, due to the cell membrane permeability. The vital DNA-specific fluorochrome (Hoechst 33342) stained all cells of the specimen. The slides were mounted in glycerol and evaluated in the same conditions as described above.

**Statistics**

For each of the studied factors, eight independent experiments were performed. Data are presented as mean±SD. The data were analyzed by Kruskal-Wallis test followed by Dunnett's Test (multiple comparison test). The calculations were done using Statistica for Windows (StatSoft). Statistical significance was accepted at p<0.05.

**RESULTS**

**Effect of IGF-I, IGF-II, EGF, SCF and TNFα on blastocyst rates and cell counts**

In the media enriched with EGF, IGF-I and IGF-II the significant (p<0.05) increase of blastocyst rate, TB and ICM cell numbers were found in comparison to controls. The effects of EGF, IGF-I and IGF-II were dose dependent (figs. 1AB, 2A). No significant effect of SCF (fig. 2B) on the examined parameters was found in medium deprived of TNFα. The viability of blastocyst cells in all experimental groups exceeded 90%. Based on these results, the concentration of 100 ng/ml was chosen for all studied treatments in the second part of the experiment (see below).

TNFα in the concentration ranging from 1 to 100 ng/ml decreased (p<0.05) the blastocyst rates and significantly reduced TB and ICM cells numbers. In
Fig. 1. Blastocyst rates and cell counts (mean±SD) following culture in medium with a/ insulin-like growth factor 1 (IGF-I), and b/ insulin-like growth factor 2 (IGF-II; n=8 independent experiments). The concentration of 100 ng/ml for IGF-I and IGF-II was chosen to be used in the second part of the study; TB: cell count of blastocyst, ICM: cell count of inner cell mass.
Fig. 2. Blastocyst rates and cell counts (mean±SD) following culture in medium with a/ epidermal growth factor (EGF), and b/ stem cell factor; (n=8 independent experiments). The concentration of 100 ng/ml for EGF was chosen to be used in the second part of the study; TB: cell count of blastocyst, ICM: cell count of inner cell mass, NS: nonsignificant (p>0.5).
media with 1000 ng/ml of TNFα only few embryos reached the blastocyst stage, therefore the statistical analysis could not be completed (fig. 3).

**Effect of IGF-I, IGF-II, EGF and SCF on embryos exposed to TNFα**

Insulin-like growth factor 1 (fig. 4A), IGF-II (fig. 4B), EGF (fig. 5A) and SCF (fig. 5B) exhibited a beneficial effect on embryo development in the presence of TNFα. The best embryo development to blastocyst stage and blastocyst quality were demonstrated in groups cultured with specific growth factors alone, followed by control groups, then groups with TNFα supplemented with growth factors and finally in groups with TNFα alone. In the experiment with SCF, ICM and TB numbers did not differ significantly between control embryos and embryos cultured with SCF alone (fig. 5B). The blastocyst cell viability in cultures with TNFα and growth factors exceeded 90%.
Fig. 4. Blastocyst rates and cell counts (mean±SD) following culture in media with tumor necrosis factor α (TNFα, 100 ng/ml) and of a/ insulin-like growth factor 1 (IGF-I, 100 ng/ml), and b/ insulin-like growth factor 2 (IGF-II; n=8 independent experiments); TB: cell count of blastocyst, ICM: cell count of inner cell mass; A: p<0.05 vs. control, B: p<0.05 vs. TNFα.
Fig. 5. Blastocyst rates and cell counts (mean±SD) following culture in media with 100 ng/ml TNFα and 100 ng/ml of A/ epidermal growth factor (EGF), and B/ stem cell factor (SCF; n=8 independent experiments); TB: cell count of blastocyst, ICM: cell count of inner cell mass; A: p<0.05 vs. control, B: p<0.05 vs. TNFα.
DISCUSSION

Mouse embryos are excellent research model as preimplantation development in this species is fairly similar to that of human embryos [38]. The cultures were done in enriched EBSS medium which is widely used in human IVF labs for the initial three days of culture. The IVF lab quality control cultures of mouse embryos in EBSS usually yield at least 60% blastocyst rates, which is comparable to control group of the current study. In the present experiment, in addition to the routine embryological evaluation, viability of blastocyst cells was analyzed [25, 48]. Since the total cell numbers of blastocyst (TB) as well as in the inner cell mass (ICM) are important predictors of embryo quality, these parameters were evaluated in previous study protocols [8, 25, 34].

The influence of EGF, IGF-I, IGF-II, SCF and TNFα on embryo development has been already evaluated in our earlier methodological study [24]. Nevertheless, in the current trial we decided to repeat those preliminary experiments to ensure the reliable comparison of embryo development in optimal culture conditions vs. culture in exposition to detrimental factor. IGF-I and IGF-II are well-proven stimulators of the pre-implantation embryo development [20, 24, 26, 44]. These growth factors not only promote the formation of blastocyst but also regulate its metabolism [17]. Favorable effect of both these factors on in vitro embryo development was also reported [16, 18, 22, 27, 39]. In the current study, both IGFs enhanced the embryo development and improved the embryo quality by increasing the TB and ICM cells numbers. The latter parameter is considered to be one of the best indicators of embryo quality [4].

EGF is a strong mitosis-promoting agent, too [12, 16, 24, 36]. Many reports demonstrated that EGF improved the preimplantation embryo development by increasing cell metabolism and proliferation. In the media enriched with EGF (and other factors of EGF family), a higher blastocyst rates as well as an augmented uptake of nutrients by blastocyst cells were found [15, 20, 21, 28, 33, 46]. An accelerated growth, improved blastocoel formation, trophoectoderm expansion and protein metabolism in the media enriched with EGF were also reported [12, 15, 46]. In in vivo conditions,
these effects are probably caused by the synergic action of all EGF receptor agonists [12]. In our study, EGF increased the blastocyst rates and positively affected TB and ICM numbers exhibiting the improvement of embryo growth and quality.

SCF exerts an anti-apoptotic effect on poorly differentiated cells such as hematopoietic progenitor cells or germinal progenitor cells [13]. The importance of SCF in the pre-implantation embryo remains mostly unknown [31]. In the current experimental setting of basic culture conditions (without detrimental factor), SCF affected neither blastocyst rate nor blastocyst cell numbers.

Since the embryotrophic potential of EGF, IGF-I and IGF-II has been determined, the question might arise whether EGF, IGF-I and IGF-II could compensate for the influence of other factors which have detrimental impact on embryo growth. SCF was included to the consecutive part of the study based on cell survival activity found in other early differentiated cells. Our previous studies confirmed such an activity of EGF, IGF-I and IGF-II toward the embryos exposed to hydrogen peroxide, a donor of free oxygen species causing oxidative stress [23]. Subsequently, we decided to investigate a putative compensative potential of EGF, IGF-I, IGF-II and SCF in cultures with a biological factor that unfavorably influence on preimplantation embryo growth. Previous reports [47, 48] as well as our own results [24] suggested that TNFα negatively affects embryo growth.

TNFα is present in the female reproductive tract [20, 45, 48] and may inhibit proliferation of embryonic cells as well as prevent formation of blastocoel. The experimental data suggest the role of TNFα in elimination of embryos showing improper structure or function. TNFα may also play a role in the induction of early spontaneous abortions [20, 35, 45, 48]. In the first part of the study we have determined a concentration of TNFα (100 ng/ml) that inhibited embryo development, allowing only few embryos to reach blastocyst stage. In addition, this concentration of TNFα was responsible for significant reduction in TB and ICM cell numbers. A ten fold higher concentration of TNFα (1000 ng/ml) caused that the majority of embryos were arrested in early stages of development and did not reach blastocyst stage. Similar results were reported previously [48].
Under normal conditions, the influence of TNFα may be balanced by the activity of survival-promoting factors. Abnormal embryo is incapable of producing factors deciding upon its survival. In such circumstances the TNFα predominance leads to the embryo death. The female reproductive tract is an additional source of survival factors. Thus, the external environment deprived of adequate amounts of stimulating growth factors could cause the inadequate embryo development secondary to relative TNFα predominance. This may also occur in in vitro embryo culture obviously deprived of survival growth factors of external origin.

Our results demonstrated the protective activity of EGF, IGF-I, IGF-II and SCF against the influence of TNFα. The effect was observed at the level of 100 ng/ml of the tested factors. The rescue of TNFα-induced apoptosis by IGFs was previously reported [5]. However, there is no such data concerning EGF and SCF. Results suggesting that TNFα effect on preimplantation embryos may be partially reversed by addition of certain growth factors to culture media are in agreement with our previous results showing the positive effect of these factors on development of embryos cultured in presence of oxidative stress induced by hydrogen peroxide [23]. The similar outcomes in two types of detrimental conditions are probably caused by different mechanisms by which TNFα and free oxygen species lead to the retardation of embryo development. It is postulated that such factors as hydrogen peroxide initiate the cell damage by the destruction of mitochondrial membranes. In contrast, TNFα acts through the receptor mediated activation of the caspases (the family of proteases involved in execution of cell death; [7, 30]). Thus, the protective potential of tested factors is probably caused not only by their antiapoptotic but also a promitotic activity.

There is no experimental model that is capable of simulating of all occurring in vivo control mechanisms responsible for survival of the preimplantation embryo. Interaction of growth factors is very complex and may be influenced by a certain stage of embryo development and hormonal milieu. Determining the concentrations of particular growth factors is another issue that should be used in in vitro models to improve the embryo development [6]. It appears that dynamic changes of concentrations of regulating factors during in vitro culture may be required. Finally, the effect of tested factors
on the survival of the embryo in sub-optimal environment could also depend on the action of many other modulating and regulating factors occurring in vivo, which were not taken into consideration in the study. Nevertheless, the stimulatory effect on embryo development of a single growth factor should encourage further studies to find practical implications in assisted reproduction techniques that would justify their utilization to protect in vitro cultured embryos from negative stimuli.

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