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Insulin and Insulin-like Growth Factor-I Promote Rabbit Blastocyst Development and Prevent Apoptosis[1]

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ABSTRACT

Insulin as well as insulin-like growth factor-I (IGF-I) promote early embryo development, and IGF-I binds to the coats of preimplantation rabbit embryos. As the IGF-I receptor is expressed from the morula stage onwards, the embryos are capable of responding to insulin and IGF-I, which is present in the oviductal and uterine secretions that surround them. The embryonic coats were removed to exclude any influence by IGF-I bound to the coats. The in vitro development of such embryos under classical conditions appears to be retarded. Addition of IGF-I (68 pM-6.8 nM) or insulin (68 nM-6.8 μM), however, promotes blastocyst formation. Embryo development under such conditions is not significantly different from that of embryos cultured with intact coats. In contrast, coat-free embryos cultured without IGF-I or insulin supplementation show apoptosis. Because IGF-I stimulates cell proliferation and prevents apoptosis, we investigated whether insulin or IGF-I may act as "survival factors" in preimplantation development. Therefore, apoptosis was induced by slight UV irradiation (254 nm wave length; 11.8 W/m²). Compared to the untreated controls, embryos displaying retarded development or degeneration were increased by 22% and 14%, respectively. Addition of IGF-I or insulin to the culture medium of UV-irradiated embryos improved [³H]thymidine incorporation and blastocyst formation significantly. By immunohistochemistry we could show that addition of insulin (0.68–68 nM) decreased apoptosis and increased cell proliferation in a dose-dependent manner, supporting blastocyst development significantly.

INTRODUCTION

Several growth factors play important roles in the control of cellular proliferation, differentiation, and morphogenesis during mammalian embryogenesis [1, 2]. Insulin and insulin-like growth factor-I (IGF-I), for example, are important factors in cellular differentiation. They are present in or are added to a wide range of culture media. In this study, we investigated the influence of insulin and IGFs on preimplantation embryo development.

IGF-I binds specifically to Day 3 rabbit embryos and their coats [3]. These coats consist of four different layers, formed by granulosa cells, oviduct and uterine secretions, and the embryo itself [4], and contain a 38-kDa IGF binding protein [3]. IGF-I has been located on the luminal epithelium of the rabbit oviduct and uterus at various stages of pregnancy [5], as it has been in other species [6-8]. Therefore, an influence of IGF-I on early rabbit embryo development, similar to that on mice [9], was postulated. In the first experiment, we tested the influence of IGF-I, IGF-II, and insulin on preimplantation embryo development. As we had shown previously that embryonic coats contain a 38-kDa IGF binding protein and that IGF-I is present on the surface of oviduct and uterine epithelial cells, we removed embryonic coats in all experiments, in contrast to previous studies, since they might contain IGF-I, which would influence the results. The results demonstrated that IGF-I and insulin promote development of coat-free rabbit embryos. In the next study, we attempted to analyze the mode of IGF-I and insulin promotion of early embryo development. In cell culture systems, IGF-I and insulin act as survival factors by suppressing apoptosis [10, 11]. We proceeded to investigate the influence of IGF-I and insulin on cell proliferation and apoptosis in preimplantation embryo development in in vitro culture.

Ki-67 was used as a marker for cell proliferation as it is present in proliferating cells throughout the entire cell cycle, except G_0 and early G_1 [12]. Immunohistological detection of Ki-67 (by MIB 1) is a reliable method, as [³H]thymidine incorporation is highly correlated to MIB 1 staining. On consecutive sections of the same embryo, apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL). To determine the effect of IGF-I and insulin as survival factors, we induced apoptosis by slight UV irradiation, which is known to induce apoptosis, while Bcl-2, induced by IGF-I [13], is able to rescue cells from apoptosis [14]. After adding IGF-I and insulin to UV-irradiated embryos, apoptosis and cell proliferation were determined.

MATERIALS AND METHODS

Animals

All experiments on animals were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals proclaimed by the Society for the Study of Reproduction and were run with the permission of the District Government at Cologne, Germany (AC 39 15/95).

Embryos were recovered from New Zealand White female rabbits ranging in age from 6 to 8 mo. They were housed individually in air-conditioned rooms (25°C, 45% relative humidity) under a 12L:12D cycle (0700–1900 h) and were fed with a commercial diet and given water ad libitum.

To induce multiple follicular growth, the rabbits received FSH-P twice daily (0.3 mg amour units s.c. per injection for 3 days; Rigaux, Segré, France). To induce ovulation, 75 IU i.v. hCG (Primogonyl; Schering, Berlin, Germany) was administered immediately after mating (i.e., 72 h after first FSH injection). Mating was accomplished by two fertile bucks. Three to six days after mating, 469 embryos were collected by flushing resected uteri with 10 ml Dulbecco's PBS (Sigma, Deisenhofen, Germany). Each experiment was conducted 2–4 times.

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Influence of IGF-I, IGF-II, and Insulin on In Vitro Embryo Development

The coats of Day 3 embryos were removed to prevent a possible influence of bound IGF-I, by incubating embryos in acidic PBS (pH 2.5) for 2 min. After washing in PBS (pH 7.3), coats were digested by 0.05% Pronase E (Serva, Heidelberg, Germany) in PBS at 37°C under visual control (darkfield, 8–15 min). Coat-free embryos were washed several times in basal salt medium/polyvinylpyrrolidone (BSM/PVP) (0.5% PVP, M_r 360 000; Sigma) by pipetting up and down to remove remaining parts of coats. Embryos were then assigned randomly to control groups and groups receiving various concentrations of IGF I, IGF II, and insulin, as follows. AI: BSM/PVP (negative control, embryos without coats), n = 20; AIIa: BSM/PVP + 68 pM IGF-I, n = 20; AIIb: BSM/PVP + 0.68 nM IGF-I, n = 20; AIIc: BSM/PVP + 6.8 nM IGF-I; n = 19; AIIIa: BSM/PVP + 68 pM IGF-II, n = 20; AIIIb: BSM/PVP + 0.68 nM IGF-II, n = 20; AIIIc: BSM/PVP + 6.8 nM IGF-II, n = 19; AIVa: BSM/PVP + 68 nM insulin, n = 20; AIVb: BSM/PVP + 0.68 μM insulin, n = 20; AIVc: BSM/PVP + 6.8 μM insulin n = 18; AV: BSM/PVP (positive control, embryos with natural coats), n = 20.

Embryos were cultured for 24 h (5% CO_2, 5% O_2 in air, 37°C) before being transferred to fresh medium containing [^{3}H]thymidine (25 μCi/ml, 925 GBq/mmol; Amersham Buchler, Braunschweig, Germany) and cultured for additional 4 h. Finally, developmental stage (morula or blastocyst) and embryonic diameter (within coats) were determined, and embryos were processed for liquid scintillation counting as described previously [15, 16].

In experiment B, the time of development of a blastocyst cavity was determined by frequent assessment (every 3 h) of the embryos. They were cultured individually for 42 h in four different groups as follow, each in 100 μl medium; because 6.8 μM insulin already displays a negative effect on embryo development (AIVc), 0.68 μM insulin was chosen for this experiment. BI: BSM/PVP (positive control, embryos with natural coats), n = 21; BII: BSM/PVP (embryos without coats + 0.68 μM insulin), n = 25; BIII: BSM/PVP (embryos without coats + 6.8 nM IGF-I), n = 21; BIV: BSM/PVP (negative control, embryos without coats), n = 22.

Assessment of Cell Proliferation by MIB 1 and Apoptosis by TUNEL

As MIB 1 has been validated only for human tissue, immunohistological detection of cell proliferation in rabbit embryos was verified by [^{3}H]thymidine incorporation, a well-accepted proliferation marker (Day 6, n = 9; three replicates). After 2 h of preincubation, 5 μCi/ml [^{3}H]thymidine was added. After an additional hour of culture, embryos were washed, embryonic coats were removed, and embryos were paraffin-embedded. Five-micrometer sections of these [^{3}H]thymidine-labeled embryos were mounted onto glass-microscopic slides and permeabilized by microwave treatment (in 10 mM citric acid, 4 × 5 min, 600 W). Endogen peroxidase was blocked by 3% H_2O_2/methanol for 20 min. After blocking of nonspecific binding sites with PBS/0.1% BSA (fraction V; Serva), sections were covered with the Ki67 antibody MIB 1 (1:40 in PBS/BSA; Dianova, Hamburg, Germany) for 1 h. The sections were then layered with the second antibody (goat anti-mouse IgG, biotinylated, 1:200 in PBS/BSA, 1 h; Jackson Immunoresearch Lab, West Grove, PA). Finally, streptavidine-peroxidase (1:200 in PBS/BSA, 30 min; Dianova) was added and detected by aminoethyl carbazole (AEC)/H_2O_2 (Histostain; Zymed, S. San Francisco, CA) under visual control. To visualize in parallel the incorporated [^{3}H]thymidine, sections were dipped subsequently into photographic emulsion (K2; Ilford, London, UK). For exposure they were stored for 6 days at 4°C in a dark box. After development of the photographic emulsion, sections were mounted with Enthelan (Merck, Darmstadt, Germany). All nuclei per section were assessed to be [^{3}H]thymidine-labeled (≥ 3 grains per nucleus after background correction), MIB 1-labeled, double-labeled, or not labeled.

The TUNEL technique has been validated before in rabbit tissue by von Rango et al. [17]. Apoptosis in embryos was detected as follows: 5-μm sections of paraffin-embedded embryos were mounted onto DNase-free microscopic slides. After endogen peroxidase was blocked by 0.3% H_2O_2/methanol (30 min), the sections were permeabilized slightly by 0.5 μg/ml Proteinase K (37°C, 10 min; Boehringer, Mannheim, Germany). In positive control sections, DNA fragmentation was induced by 15-min DNase treatment (3 U/ml, 37°C; Boehringer). Preincubation was performed in TdT buffer (30 min, 37°C; MBI Fermentas, Vilnius, Lithuania), followed by incubation in TdT buffer containing 0.3 mM deoxy (d)ATP/μl, 30 μM digoxigenin-labeled dUTP/μl, and 0.13 U TdT/μl (1 h, 37°C; Boehringer; negative control without digoxigenin-dUTP). Sections were washed 2 × 10 min in double-strength SSC (single-strength SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and then incubated with peroxidase-labeled anti-digoxigenin (1:300 in TRIS/NaCl + 1% blocking reagent, 45 min; Boehringer). Labeling was detected by AEC/H_2O_2 (Histostain; Zymed) under visual control.

Influence of UV Irradiation on In Vitro Embryo Development

In experiment C, to induce controlled apoptosis, embryos were UV-irradiated (5.9 W/m^2 per second, 254 nm wave length; universal UV lamp, Camag, Berlin, Germany). Day 3 embryos were preincubated for 2 h in PBS/PVP. After exposure to UV light for either 30 sec (177 W/m^2, n = 19, group CI) or 2 sec (11.8 W/m^2, n = 18, group CII), embryos were cultured in fresh BSM/PVP. Culture medium was changed to avoid active oxygen radicals generated within by UV light. Parallel to this, untreated embryos were cultured as controls. Embryos were assessed for their development after 22 h more (degenerated, retarded, or blastocyst).

Influence of IGF-I and Insulin on In Vitro Development of UV-Irradiated Embryos

Day 4 embryos were divided randomly into groups, as follows, for experiment D after 1 h preincubation. DI: BSM/PVP (negative control; coat-free, UV-irradiated), n = 15; DIIa: 68 pM IGF-I in BSM/PVP (coat-free, UV-irradiated), n = 14; DIIb: 6.8 nM IGF-I in BSM/PVP (coat-free, UV-irradiated), n = 15; DIIIa: 0.68 nM insulin in BSM/PVP (coat-free, UV-irradiated), n = 13; DIIIb: 68 nM insulin in BSM/PVP (coat-free, UV-irradiated), n = 12; DIV: BSM/PVP (positive control; coat-free, without UV irradiation), n = 15.

We decreased the dosage of insulin (compared to that in experiment A), as amounts of more than 680 nM insulin have a negative effect on embryo development. The influence of IGF-II was not assessed in this experiment, as it

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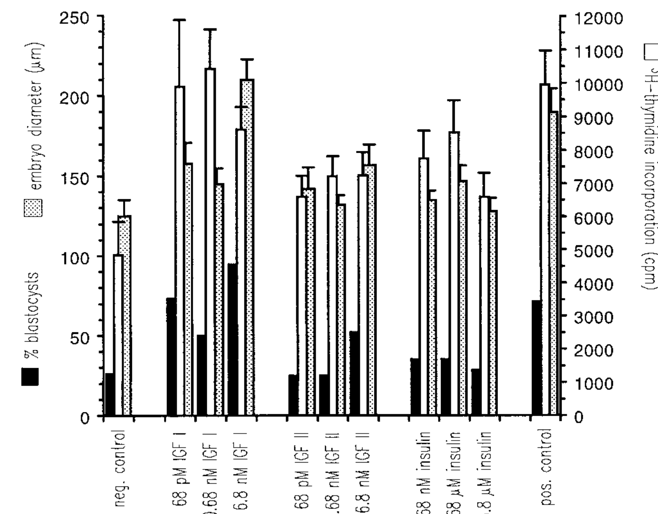

^{3}H-thymidine incorporation (above diagonal); embryonic diameter (below diagonal)

	AI	AII a	AII b	AII c	AIII a	AIII b	AIII c	AIV a	AIV b	AIV c	AV
AI		**	**	**	n.s.	*	n.s.	*	*	n.s.	**
AII a	*		n.s.	n.s.	*	*	n.s.	n.s.	n.s.	*	n.s.
AII b	n.s.	n.s.		**	*	*	**	n.s.	n.s.	**	n.s.
AII c	**	*	**		*	n.s.	n.s.	n.s.	n.s.	*	n.s.
AIII a	n.s.	n.s.	n.s.	**		n.s.	n.s.	n.s.	n.s.	n.s.	**
AIII b	n.s.	*	n.s.	**	n.s.		n.s.	n.s.	n.s.	n.s.	*
AIII c	*	n.s.	n.s.	**	n.s.	n.s.		n.s.	n.s.	n.s.	*
AIV a	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.
AIV b	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.
AIV c	n.s.	*	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.		*
AV	**	n.s.	*	n.s.	*	**	n.s.	**	*	**	

embryonic diameter

FIG. 1. Development of coat-free Day 3 embryos shown as percentage of blastocysts formed, embryonic diameter (μm), and cell proliferation ([^{3}H]thymidine incorporation as cpm). AI) Coat-free embryos, negative control. AV) Embryos with intact coats, positive control. AII) Coat-free embryos cultured with IGF-I. AIII) Coat-free embryos cultured with IGF-II. AIV) Coat-free embryos cultured with insulin. For full description of treatments and sample sizes, see *Materials and Methods*. * $p < 0.05$, ** $p < 0.01$).

had been demonstrated in experiment A that IGF-II affected embryo development only slightly. Groups DI–DIIIb were UV-irradiated for 2 sec (11.8 W/m^2) and were transferred into fresh medium. After a culture period of 23 h, 5 μCi/ml [^{3}H]thymidine was added for 4 h more. Finally, the embryonic diameter and developmental stage were assessed, and embryos were processed for liquid scintillation counting to determine [^{3}H]thymidine incorporation as an expression of cell proliferation.

Influence of Insulin and IGF-I on Cell Proliferation (MIB 1) and Apoptosis (TUNEL) in UV-Irradiated Embryos

As insulin is widely used in cell and embryo cultures and it promotes embryo development as well as IGF-I, we tested the influence of various concentrations of insulin on apoptosis and cell proliferation (experiment E). Coat-free Day 4 embryos were divided randomly into groups as follows. EI: 68 nM insulin in BSM/PVP, n = 3; EII: 6.8 nM insulin in BSM/PVP, n = 3; EIII: 0.68 nM insulin in BSM/PVP, n = 3; EIV: BSM/PVP (negative control), n = 3.

After 1-h preincubation and UV irradiation for 2 sec (11.8 W/m^2), embryos were incubated for a further 6 h in fresh medium. Embryos were paraffin-embedded, and cell proliferation (MIB 1) and apoptosis (TUNEL) were determined on 5-μm sections.

Furthermore, the following 3 groups of Day 3 embryos were cultured for 24 h, as described in experiment A, and paraffin-embedded: 1) positive control (cultured within their natural coats, like group A V; n = 2), 2) negative control (coat-free embryos cultured serum-free, like group A I; n = 2), and 3) coat-free embryos cultured in the presence of 6.8 nM IGF-I, as in group AIIc (n = 2). Five-micrometer sections were mounted onto glass-microscopic slides and evaluated for cell proliferation (MIB 1) and apoptosis (TUNEL).

Statistics

Overall effects of treatments were evaluated by ANOVA or paired t-test (influence of insulin and IGF-I on cell proliferation (MIB 1) and apoptosis (TUNEL) in UV-irradiated embryos). ANOVA was followed by Scheffe's F test to evaluate specific differences. Data are presented as mean ± SEM.

RESULTS

Influence of IGF-I, IGF-II, and Insulin on In Vitro Embryo Development

Compared with the negative control, IGF-I increased the number of morulae that developed to blastocysts by a factor of 2 to 3 as well as increasing embryonic diameter and cell proliferation as shown by [^{3}H]thymidine incorporation (Fig. 1). In general, there was no significant difference between coat-free embryos cultured with IGF-I and the positive control.

IGF-II increased the number of blastocysts and embryo diameter only at a concentration of 6.8 nM, but compared to the positive control, embryo development still was retarded. Insulin at 6.8 μM had no effect compared to the negative control, while lower insulin concentrations (0.68 μM and 68 nM) significantly increased cell proliferation. Between 0.68 μM and 68 nM insulin, and between IGF-I and the positive control, there was no significant difference.

We can conclude that IGF-I significantly enhanced the development of coat-free embryos, while the same concentration of IGF-II was less effective. Insulin showed a positive effect only at a 10- to 100-fold higher concentration.

In an additional experiment, the time of blastocyst formation was investigated. As shown in Figure 2, development was retarded by 15 h in the negative versus positive control. After 42 h of culture, only 45% of the embryos had developed to blastocysts, compared to 95% in the positive control. Addition of IGF-I or insulin improved blastocyst formation. Although in the insulin group blastocysts were observed earlier, embryos in the IGF-I group did catch up later on. In total, all 4 curves run in parallel: the positive control on top, negative control at the bottom, insulin and IGF-I in between (Fig. 2).

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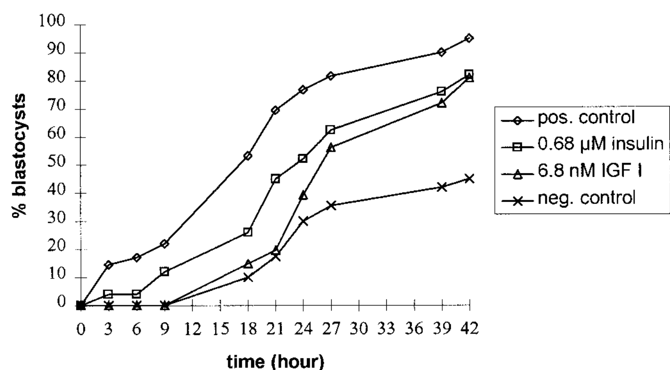

FIG. 2. Embryonic development shown as percentage of blastocysts formed by coat-free Day 3 embryos (negative control, n = 22), compared to coat-free embryos cultured in the presence of IGF-I (n = 21) or insulin (n = 25) and those cultured with intact coats (positive control, n = 21).

Assessment of Cell Proliferation by MIB 1 and Apoptosis by TUNEL

To evaluate the immunohistological assessment of cell proliferation by MIB 1 in rabbit embryos, we performed double labeling of dividing cells with [^{3}H]thymidine and MIB 1. [^{3}H]Thymidine incorporation was detected by autoradiography after immunohistological detection of Ki-67 by MIB 1. All nuclei per section were evaluated for being MIB 1- and/or [^{3}H]thymidine-positive.

Figure 3 shows that 77.5 ± 3% of all nuclei were both ^{3}H- and MIB 1-positive, i.e., 98% of all ^{3}H-labeled nuclei were also positive for MIB 1. Therefore, a high correlation between MIB 1 staining and [^{3}H]thymidine incorporation (r = 0.436, n = 27; $p < 0.001$) can be stated, indicating that MIB 1 is highly reliable for detection of cell proliferation in rabbit embryos.

Furthermore, detection of apoptosis by TUNEL was evaluated by DNase-induced DNA fragmentation. On DNase-treated sections, 100% of all nuclei revealed a high number of DNA fragments (as seen in apoptotic nuclei). In negative control sections (non-digoxigenin-labeled dUTP), there was no staining visible.

Influence of UV Irradiation on In Vitro Embryo Development

UV light induces apoptosis. In this experiment, the dosage inducing apoptosis but not leading to total embryo degeneration was determined. After 30 sec of UV irradiation (177 W/m^2), all embryos degenerated within the following 24 h of culture. Two seconds of UV irradiation (11.8 W/m^2) resulted in 38.1% normally developed, 22% retarded (morulae), and 38.9% degenerated embryos. In the control group (also coat-free), 75% of all embryos developed to blastocysts, while 25% degenerated. Two seconds of UV irradiation induced a slight but detectable amount of apoptosis, causing an 36% increase in retarded and degenerated embryos (Fig. 4).

Influence of IGF-I and Insulin on In Vitro Development of UV-Irradiated Embryos

IGF-I and insulin, shown in experiment A to improve embryo development, were investigated regarding their influence on in vitro development of UV-irradiated embryos (2 sec, 11.8 W/m^2). Both IGF-I and insulin improved embryo development (83.3–100% blastocysts vs. 44.4% in negative control; Fig. 5), cell proliferation, and embryo diameter significantly in both dosages. Cell proliferation was comparable to that of the positive control; with addition of insulin it tended to be even higher. We can conclude that IGF-I and insulin were capable of overcoming the negative effect of UV irradiation on embryo development. The effect of insulin, however, tended to be greater than that of IGF-I.

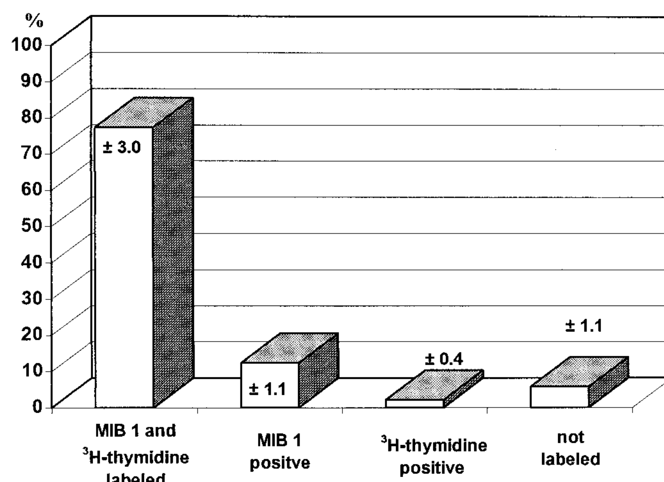

FIG. 3. Evaluation of the detection of cell proliferation in Day 6 embryos by MIB 1 and [^{3}H]thymidine incorporation (n = 9, 3 replicates each).

Influence of Insulin on Cell Proliferation (MIB 1) and Apoptosis (TUNEL) in UV-Irradiated Embryos (11.8 W/m^2)

Because insulin is widely used in embryo and cell cultures (much more often than IGF-I) and has been shown to be the more potent agent in decreasing the negative influence of UV irradiation on embryo development, we conducted the last experiment with insulin alone. Cell proliferation was detected immunohistologically using a Ki-67 antibody (MIB 1), and apoptosis was determined using the TUNEL method. UV irradiation (11.8 W/m^2) resulted in a high amount of apoptosis (86.8%) whereas hardly any cell proliferation (4.1%) was detectable (Fig. 6 and Fig. 7, c and d). Addition of insulin in increasing amounts decreased apoptosis and increased cell proliferation in a dose-dependent manner (Fig. 6 and Fig. 7, e and f). Adding 68 nM insulin to UV-irradiated embryos resulted in a restoration of cell proliferation (91.2% vs. 4.1% in negative control) and reduced apoptosis (5.3% vs. 86.8%).

Coat-free Day 3 embryos cultured 24 h in serum-free medium with or without IGF-I (6.8 nM) were evaluated for

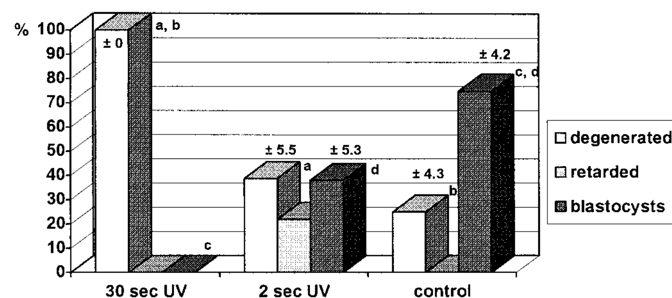

FIG. 4. Development of Day 3 embryos expressed as percentage of embryos developed to blastocysts, persisting as morulae (retarded), or degenerated, after 30 sec (177 W/m^2, n = 19) or 2 sec (11.8 W/m^2, n = 18) of UV irradiation (254 nm wave length); control, n = 12; a, b, c, $p < 0.01$; d, $p < 0.05$.

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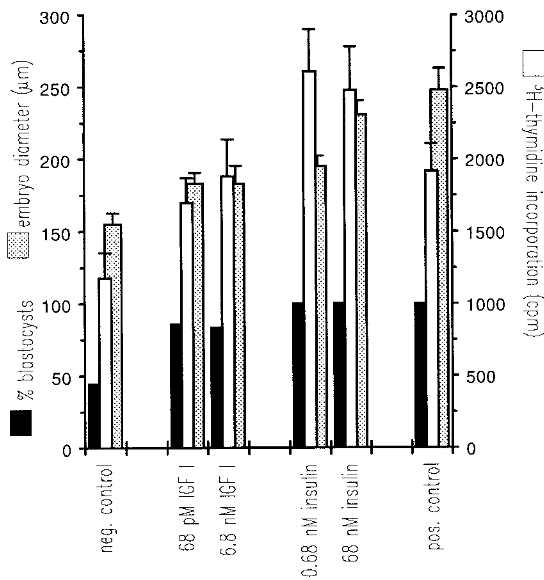

^{3}H-thymidine incorporation

	DI	DII a	DII b	DIII a	DIII b	DIV
DI		*	*	**	**	**
DII a	*		n.s.	*	*	n.s.
DII b	*	n.s.		n.s.	n.s.	n.s.
DIII a	**	n.s.	n.s.		n.s.	n.s.
DIII b	**	**	**	n.s.		n.s.
DIV	**	**	**	**	n.s.	

embryonic diameter

FIG. 5. Development of coat-free Day 3 embryos after UV irradiation (11.8 W/m^2) shown as percentage of formed blastocysts, embryonic diameter (µm), and cell proliferation ([^{3}H]thymidine incorporation as cpm). DI) Coat-free embryos, negative control. DIV) Embryos with intact coats, positive control. DII) Coat-free embryos cultured with IGF-I. DIII) Coat-free embryos cultured with insulin. For full description of treatments and sample sizes, see *Materials and Methods*. * $p < 0.05$, ** $p < 0.01$.

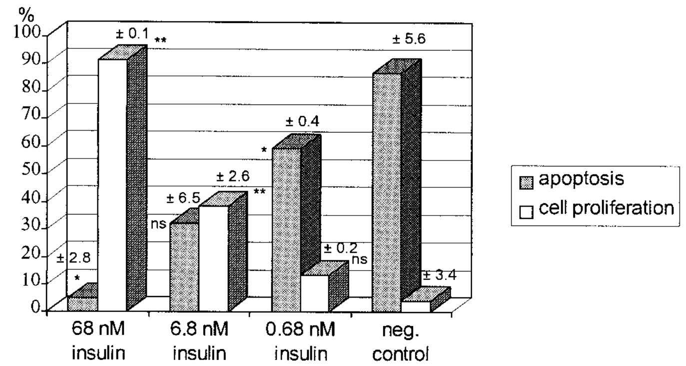

FIG. 6. Percentage of proliferating cells (MIB 1) and apoptotic cells (TUNEL) in UV-irradiated coat-free Day 4 embryos, treated with different amounts of insulin, n = 12. * $p < 0.05$, ** $p < 0.01$).

cell proliferation (MIB 1) and apoptosis (TUNEL). Positive control embryos cultured with their natural coats developed to blastocysts, showing 100% cell proliferation and no apoptosis (Fig. 8a). In contrast, coat-free embryos cultured serum-free and without IGF-I, which seldom develop to blastocysts, exhibited apoptotic cells. After addition of IGF-I to culture medium, no apoptosis was detectable (Fig. 8c). It can be concluded, that IGF-I and insulin were able to promote embryo development by decreasing apoptosis and increasing cell proliferation.

DISCUSSION

IGF-I and insulin support early embryonic development by decreasing apoptosis and increasing cell proliferation. The presence of IGF-I as well as insulin receptors in preimplantation embryos has been described in several reports. IGF-I and insulin receptor mRNA is present in mouse embryos from the 8-cell stage onwards [18, 19]; the receptor is present in compacted 8-cell embryos [20]. In the mouse and rabbit, insulin and IGF-I bind to embryos from the morula stage onwards [3, 21]; binding sites have been shown in the trophoblast as well as in the embryoblast [3, 22, 23]. The question whether or not IGF-I is synthesized by preimplantation embryos is controversial. While some investigators were unable to detect IGF-I mRNA in preimplantation embryos [18, 24], others described its presence from the oocyte stage to the blastocyst stage [25, 26]. The protein has not been shown to be synthesized by preimplantation embryos. Therefore, it has been proposed that IGF-II, which is known to be produced as early as the two-cell stage, acts via the IGF 1 receptor [18, 27, 28]. On the other hand, maternal IGF-I is present in oviductal and uterine secretions surrounding the embryo at all times. IGF-I is detectable in the oviduct [5, 7, 29, 30] as well as uterus [5, 31-34], and in their respective secretions [6, 8, 35]. In fowl, such as the chicken, maternal IGF-I is present in the egg yolk and is thereby available to early embryos [36]. IGF-I is bound to rabbit embryonic coats via a specific 38-kDa binding protein [3], while insulin is accumulated (in a nonspecific manner) by the mouse zona pellucida [23]. High-resolution electron microscopy has visualized the uptake of insulin and IGF-I by specific receptors located on microvilli of trophectoderm cells in mouse blastocysts [22, 23, 37]. These studies demonstrate that IGF-I and insulin are internalized and transported towards the yolk sac. Furthermore, in other species, like the horse, the uptake of maternal IGF-I and transport towards the yolk sac could be demonstrated in late blastocysts [38]. Although a growth-promoting effect via the IGF-I receptor could be observed only in vivo in null mutant mice after day 11 of gestation [28], several effects on in vitro development of much younger embryos have been described. IGF-I and insulin stimulate glucose uptake [39, 40] and protein synthesis in preimplantation mouse embryos [22, 26]. IGF-I increases the cell number of mouse blastocysts [26], in particular the inner cell mass [9]. Therefore, IGF-I increases embryonic metabolism and cell proliferation, influencing in vitro embryo development positively. The influence on blastocyst formation, as an easy detectable sign of "in-time" embryo development, has been controversial. While Harvey and Kaye [9] noted an increase in the frequency of blastocyst formation, others [37, 41] did not. The reason for such contradictory results may be varying amounts of maternal IGF-I bound to the coats. Consequently, we removed the embryonic coats in all experiments to eliminate a possible contamination. Under these conditions, we detected improved embryonic growth in the presence of IGF-I and insulin.

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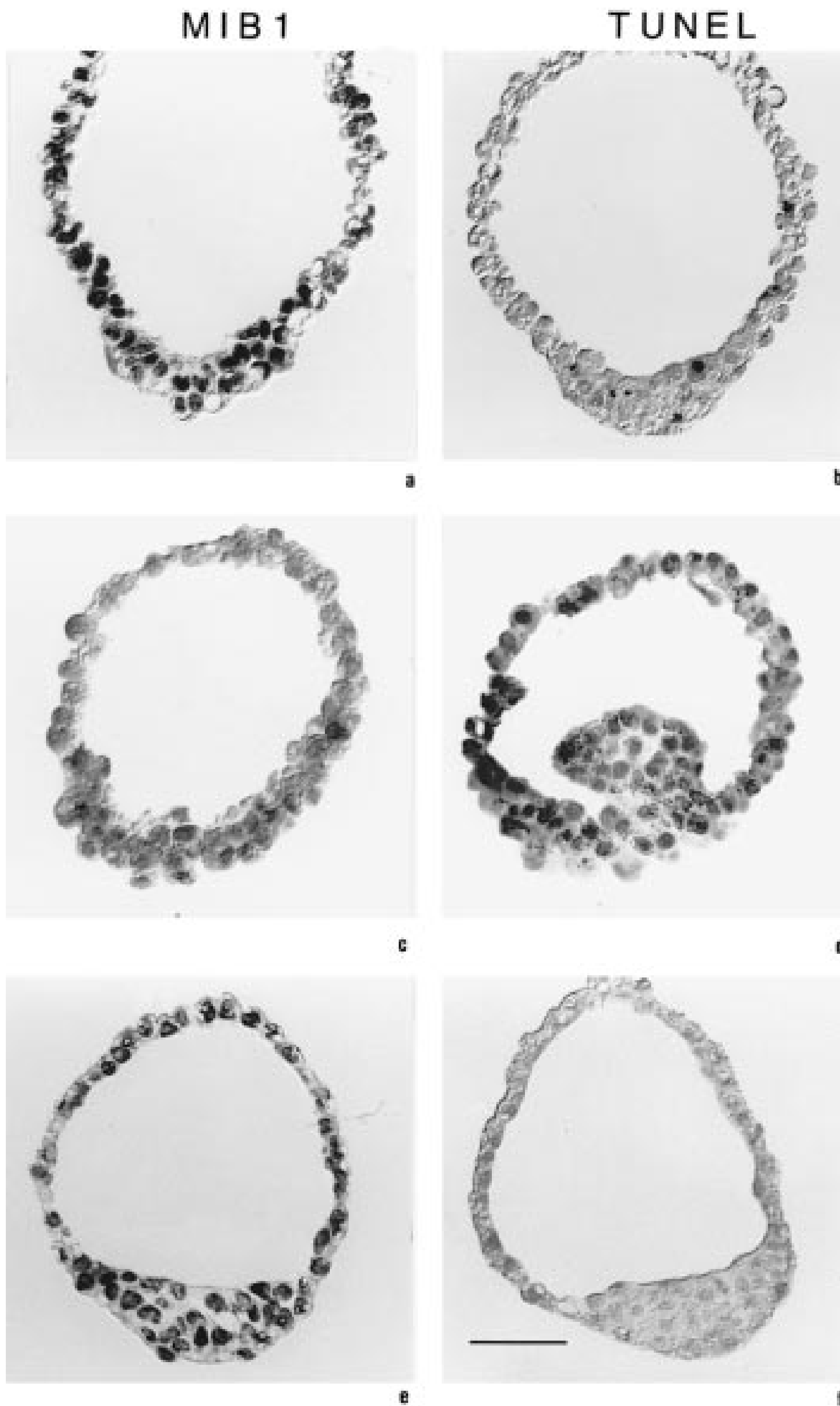

FIG. 7. Cell proliferation (MIB 1; panels on left) and apoptosis (TUNEL; panels on right) of UV-irradiated coat-free Day 4 embryos. **a, b**) Positive control, normal Day 4 embryo; **c, d**) negative control, coat-free embryo, UV-irradiated; **e, f**) coat-free embryo UV-irradiated, treated with 68 nM insulin. Bar represents 50 μm.

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[^{3}H]Thymidine incorporation, embryonic diameter, and blastocyst formation was increased significantly by 68 pM, i.e., 0.5 ng/ml, IGF-I, which is similar to the concentrations used by Harvey and Kaye [9]. Furthermore, we investigated a new mode of action for the promotion of early embryo development by insulin/IGF-I. Insulin and IGF-I were capable of acting in preimplantation embryos as survival factors by reducing apoptosis and increasing cell proliferation. That insulin and IGF-I act as survival factors has been discussed for several cell culture systems, such as oligodendrocytes [42], fibroblasts [10], epithelial cells [11], teratocarcinoma cell lines [43], and BALB/c 3T3 [44]. To test this hypothesis, we induced apoptosis by 11.8 W/m^2 UV irradiation, causing an 36% increase in retarded and degenerated embryos. In former studies, it has been demonstrated that UV light decreases embryo development [16] by caus-

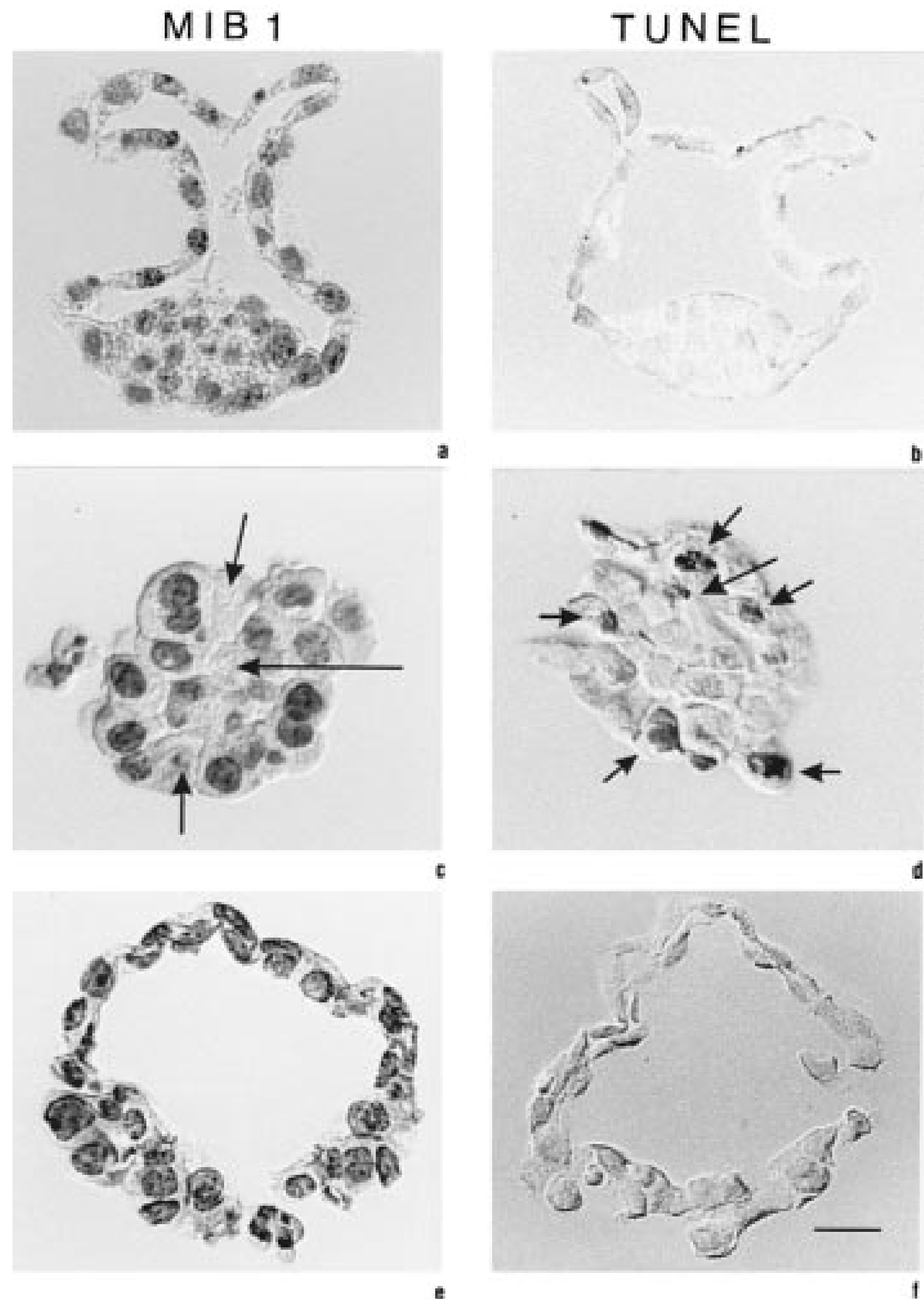

FIG. 8. Cell proliferation (MIB 1; panels on left) and apoptosis (TUNEL; panels on right) in Day 3 embryos cultured for 24 h. **a, b**) Positive control, embryos cultured with their natural coats; **c, d**) negative control, coat-free embryos cultured in serum free medium; **e, f**) coat-free embryos cultured serum-free in the presence of 6.8 nM IGF-I. Arrows in **c** point to the nuclei that are MIB 1 negative; in **d**, to those that are undergoing apoptosis. Bar represents 20 μm.

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ing DNA damage (60 W/m^2, [45]). One cause of DNA damage and subsequent apoptosis [46, 47] is reactive oxygen species (ROS; oxygen radicals) induced by UV irradiation [48-50]. IGF-I has been shown to be antioxidative [51] by inducing Bcl-2 and Bcl-X_L via the IGF 1 receptor [13, 52]. Insulin and IGF-I antagonize apoptosis by inhibiting ROS generation [53, 54]. These results explain why IGF-I and insulin, which are widely added directly or indirectly to embryo culture media, influence embryo development positively. Improved embryonic development by addition of IGF-I or insulin has been described by several investigators [5, 9, 55-58]. Furthermore, both are added indirectly by supplementing culture media with serum containing IGF-I bound to its carrier IGF binding protein-3 [59], BSA (D. Schams, personal communication 1994), or uterine fluids [8, 60, 61]. Certain coculture systems (granulosa cell coculture [62-64], oviductal cell coculture or conditioned medium [7, 29, 35, 65-68], uterine cells [67, 69], and vero cells [69, 70]) improve early embryo development, perhaps by providing embryos with insulin and IGFs.

We have demonstrated that insulin and IGF-I support early embryonic development by preventing apoptosis and by increasing cell proliferation. This might be an in vitro effect, protecting embryos against hazards caused by inadequate culture conditions, such as exposure to UV light and ROS. However, a similar effect of IGF-I has also been proposed for in vivo preimplantation embryo development [56]. In general, addition of insulin or IGF-I to the microenvironment of embryos proves to be beneficial for blastocyst development.

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