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Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-α in vitro

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SUMMARY

The morphogenetic function of the transient phase of cell death that occurs during blastocyst maturation is not known but it is thought that its regulation results from a delicate balance between survival and lethal signals in the uterine milieu. In this paper, we show that blastocysts from diabetic rats have a higher incidence of dead cells than control embryos. Differential lineage staining indicated that increased nuclear fragmentation occurred mainly in the inner cell mass. In addition, terminal transferase-mediated dUTP nick end labeling (TUNEL) demonstrated an increase in the incidence of non-fragmented DNA-damaged nuclei in these blastocysts. Analysis of the expression of clusterin, a gene associated with apoptosis, by quantitative reverse transcription-polymerase chain reaction detected an increase in the steady-state level of its transcripts in blastocysts from diabetic rats. In situ hybridization revealed that about half the cells identified as expressing clusterin mRNA exhibited signs of nuclear fragmentation. In vitro experiments demonstrated that high D-glucose increased nuclear fragmentation, TUNEL labeling and clusterin transcription. Tumor necrosis factor-α (TNF-α), a cytokine whose synthesis is up-regulated in the diabetic uterus, did not induce nuclear fragmentation nor clusterin expression but increased the incidence of TUNEL-positive nuclei. The data suggest that excessive cell death in the blastocyst, most probably resulting from the overstimulation of a basal suicidal program by such inducers as glucose and TNF-α, may be a contributing factor of the early embryopathy associated with maternal diabetes.

Key words: blastocyst, cell death, clusterin, diabetes, glucose, rat, TNF-α

INTRODUCTION

It has long been recognized that many critical developmental events occur through the death of selected cells in predictable places and at predictable times (Glucksmann, 1951; Sanders and Wride, 1995). The fact that these cells appear to die naturally led to the proposal that their elimination results from the operation of a physiological procedure which was designated 'programmed cell death' (Wyllie et al., 1980). Active cell elimination appears to be engaged very early in development since the process of proamniotic cavity formation already relies on the destruction of selected cells (Coucouvanis and Martin, 1995). Several reports also suggest that this eliminative process may be activated even earlier, at the blastocyst stage. Following the initial description of dead cells in preimplantation embryos (Potts and Wilson, 1967), additional evidence has been based on electron microscopy (El-Shershaby and Hinchliffe, 1974), reconstruction of serial sections (Copp, 1978) and differential labeling with DNA fluorochromes (Handyside and Hunter, 1986). In mouse blastocysts, cell death occurs as they reach maximal expansion, with most disintegrating cells located in the inner cell mass (ICM) lineage. It has been hypothesized that the primary function of this process is to allow for the removal of redundant or defective cells from the blastocyst, hence eliminating the risk of maintaining a subgroup of cells with unwanted developmental potential within the embryonic germ layers (Pierce et al., 1989).

It is not clear whether the elimination process observed in blastocysts is cell autonomous or controlled by extraembryonic signals. Mouse blastocysts rendered unresponsive to both intracellular and extracellular survival signals simultaneously have been shown to engage in massive cell death, suggesting that embryonic cells are equipped with a death-by-default mechanism that requires constant suppression (Weil et al., 1996). One uterine survival agent may be transforming growth factor-α. This effector is expressed in the pregnant mouse uterus at the time of implantation (Paria et al., 1994) and its addition to the composition of culture medium reduces the incidence of dead cells in mouse blastocysts in vitro (Brison and Schultz, 1997). Thus, if the normal programming of developmental genes, including those involved in cell death, depends on the correct integration of multiple signals, it seems reasonable to speculate that, under certain pathological conditions, an imbalance in these signals would lead to inappropriate cell determination (Kimmel et al., 1993). In addition, because the correct allocation of cells to the ICM is of funda-

mental importance for the subsequent formation of the embryonic germ layers (Tam, 1988), it can be postulated that excessive cell death in this lineage at the blastocyst stage would predispose to later developmental deficiencies.

Experiments on animal models have shown that the developmental status of embryos recovered at the blastocyst stage from diabetic females is markedly altered (Pampfer and De Hertogh, 1996). Rat blastocysts from streptozotocin-induced diabetic rats contain fewer cells than control embryos (Vercheval et al., 1990) and this cellular loss is predominantly located in the ICM (Pampfer et al., 1990a). Recent investigations on blastocysts from genetically prone diabetic BB/E rats have confirmed both the cellular deficit and the higher susceptibility of the ICM (Lea et al., 1996). In addition, when blastocysts from streptozotocin-induced diabetic rats are incubated in vitro, their viability continues to decline in contrast to control blastocysts (Pampfer et al., 1994a), suggesting that the impact of the maternal disorder is long-lasting. Because diabetes is associated with a myriad of hormonal, metabolic and other alterations, identifying which imbalanced factor(s) are responsible for the developmental anomalies detected before implantation may prove difficult. Although high glucose concentrations are likely to be accumulated in the uterine lumen of the diabetic female (Nilsson et al., 1980) and high glucose is detrimental to rat blastocysts (De Hertogh et al., 1991), recent data suggest that more indirect effects may be involved. Previous observations that tumor necrosis factor-α (TNF-α) is up-regulated in the diabetic uterus (Pampfer et al., 1995a, 1997) combined with findings that TNF-α inhibits rat blastocyst growth (Pampfer et al., 1994b) support the hypothesis that inappropriate release of this cytokine may be integral in inducing the early embryopathy associated with maternal diabetes.

In the present work, we used different cellular and molecular techniques to detect an increase in the incidence of cell death in blastocysts recovered from diabetic rats and in normal blastocysts incubated with high glucose and/or TNF-α.

MATERIALS AND METHODS

Animals and embryos

In experiments involving diabetic rats, adult females were administered a single injection of streptozotocin and tested 24 hours later for their glycosuria (Vercheval et al., 1990). 2-4 weeks after drug treatment, diabetic females were mated to normal males and pregnancy was determined on the next morning by the presence of a vaginal plug (gestational day 1). Normal pregnant females were used in parallel as controls. On day 5 or day 6, the uterine horns from diabetic and control rats were flushed to recover the embryos. Only those embryos displaying the morphology of mid-expanded blastocyst on day 5 or hatched blastocyst on day 6 were selected for further study. Whole blood glycemia was measured using a glucose test strip meter. Serum levels of β-hydroxybutyrate were measured using a colorimetric assay (ref. 310-A, Sigma, St Louis, USA) and serum concentrations of TNF-α were measured by means of an enzyme-linked immunosorbent assay (ref. 80-3905-01, Genzyme, Boston, USA). For in vitro experiments, blastocysts were recovered from normal rats on day 5 and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 hours. The basal culture medium was Ham’s F-10 (ref. 04-1040-20, Gibco, Buckinghamshire, UK) supplemented with 1 mM glutamine, 0.1% bovine serum albumin, 100 U/ml penicillin and 100 µg/ml streptomycin. Depending on the experiments, various combinations of glucose (control 6 mM D-glucose, 17 mM D-glucose, 6 mM D-glucose + 11 mM L-glucose) and/or 3000 U/ml of rat recombinant TNF-α (ref. CY-044, Innogenetics, Zwijnaarde, Belgium) were added to the culture medium. The specificity of TNF-α action was confirmed using a neutralizing rabbit anti-rat TNF-α antibody (ref. CY-051, Innogenetics) in 100-fold molar excess.

Differential nuclear staining

The number of cells per embryo and their distribution between the inner cell mass (ICM) and the trophectoderm (TE) were counted using a differential staining technique based on the partial immunolysis of the outer TE layer and the exposure of the embryo to two DNA dyes with different permeability and fluorescent properties (Pampfer et al., 1990b). Partial complement-mediated lysis of the peripheral TE layer resulted in the permeabilization of that lineage to propidium iodide (PI, which is normally excluded from membrane-intact cells) whereas bisbenzimide (Hoechst 33258, HO) entered into all the cells of the embryo, including the ICM. Nuclei in the ICM were found clustered at the center of the embryo and stained blue under u.v. light whereas nuclei of TE cells looked more spread, larger in size and stained pink. In both cell lineages, some nuclei appeared fragmented or scattered into highly fluorescent chromatin particles which were still comprised within intact cell boundaries as determined under visible light. For each blastocyst, the incidence of disintegrating nuclei in the two cell lineages was expressed as percentage of the corresponding numbers of ICM and TE cells (nuclear fragmentation index). In a limited set of experiments, rat blastocysts were exposed to the two DNA dyes without prior treatment with pronase and complement. The observation of cells containing either HO-stained or PI-stained fragmented nuclei was restricted to the TE layer in these embryos.

Terminal transferase-mediated dUTP nick end labeling (TUNEL)

Nuclei containing abundant 3′-hydroxyl termini were detected using the TUNEL technique (Gavrieli et al., 1992). Blastocysts were exposed to 0.4% pronase, fixed in 4% paraformaldehyde in PBS, exposed to 0.3% hydrogen peroxide in methanol and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. Following a rinse in PBS supplemented with 0.5% Tween-20 (PBS-T), the embryos were prestained with 25 µg/ml of HO in PBS-T. Incubation with 50 units/ml of terminal transferase and 15 µM of fluorescein-dUTP was performed for 35 minutes at 37°C in 50 µl drops. The embryos were exposed to sheep anti-fluorescein antibody conjugated to peroxidase and nuclear TUNEL staining was developed in a solution of diaminobenzamide and nickel chloride. Exposure of the embryos to u.v. light allowed to visualize and count the total number of HO-nuclei and the proportion of fragmented nuclei. Observation under visible light allowed for the counting of TUNEL-positive nuclei. For each blastocyst, the incidence of fragmented nuclei (F-index), TUNEL-positive nuclei (T-index) and nuclei with both labeling features (F+T-index) was calculated as percentage of the total cell number. For negative control experiments, the terminal deoxynucleotidyl transferase was omitted from the labeling reaction. For positive control reactions, blastocysts were pretreated with 15 units/ml of deoxyribonuclease I before labeling.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The steady-state level of clusterin transcripts was assessed by means of a quantitative RT-PCR method based on the co-amplification of the unknown amount of clusterin cDNA in each sample with a known amount of internal standard. To construct the standard, a 564 bp-long amplicon was generated from rat placental cDNA with two primers (SP1 and SP2) specific for clusterin (Fig. 1). The genomic structure of rat clusterin and its cDNA sequence (GenBank M64723) have been reported (Wong et al., 1993). The amplicon was inserted

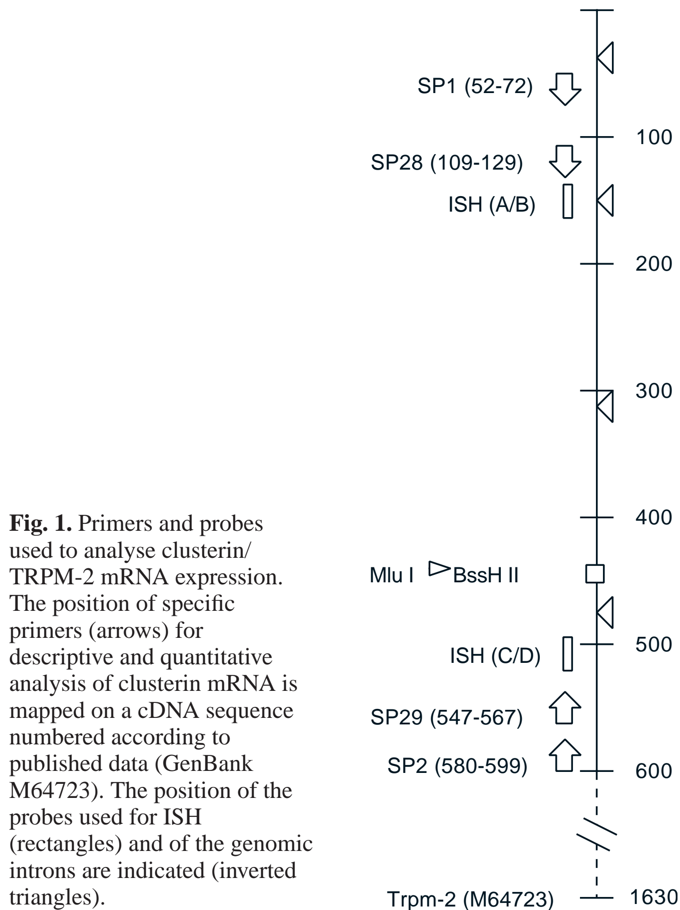

Fig. 1. Primers and probes used to analyse clusterin/ TRPM-2 mRNA expression. The position of specific primers (arrows) for descriptive and quantitative analysis of clusterin mRNA is mapped on a cDNA sequence numbered according to published data (GenBank M64723). The position of the probes used for ISH (rectangles) and of the genomic introns are indicated (inverted triangles).

into a pCRII plasmid (clone p16rCLUn) and modified to increase its length by 4 nucleotides. One of the mutated constructs (p9rCLUm) was used as internal standard for PCR. To study clusterin mRNA expression in blastocysts, total RNA was extracted from pools of 30 embryos and retrotranscribed with 200 µM random hexamers and MMLV reverse transcriptase (100 U per reaction) (Pampfer et al., 1994b). A fixed amount of internal standard (5×10^5 molecules) was added to each cDNA sample which was then serially diluted in PCR reaction mixture. Each dilution series (1:1, 1:3 and 1:9) was amplified with 1 µM of primers SP28 and SP29 (Fig. 1) and Taq DNA polymerase (5 U per reaction). PCR reactions were terminated after 32 cycles when the amplification process was still within the exponential phase so that the ratio of unknown target to known internal standard at the initiation of the reaction was conserved. Aliquots of PCR reactions were electrophoresed on 5% polyacrylamide gel containing 8 M urea. Silver-stained amplicons produced from the target cDNA (459 bp) and the internal standard (463 bp) were quantitated by direct scanning densitometry. For each cDNA sample, the ratio between the integrated intensities of the target and the internal standard was calculated at the three dilutions tested and the average ratio value was expressed in arbitrary units. Two control cDNAs, one from a group of clusterin negative rat preblastocyst embryos (morula stage) and one from clusterin positive rat ovaries, were serially diluted after addition of internal standard and run in parallel in each assay, together with a mock sample prepared without RNA input. The blastocyst cDNAs were also tested for variations in the retro-transcription step by amplifying the samples with primers complementary to nucleotide positions 273-292 (SP30) and 552-571 (SP23) of the rat β-actin cDNA sequence (Nudel et al., 1993). Corrections made to the clusterin expression values never exceeded 10% of the original result.

In situ hybridization (ISH)

Localization of clusterin mRNA expression in rat blastocysts was studied by ISH using specific 5′-end biotinylated probes. A combination of two oligonucleotides (antisense ISHB and ISHD) complementary to distinct regions of the clusterin cDNA sequence was used against a combination of corresponding reverse oligonucleotides (sense ISHA and ISHC) as controls (Fig. 1). Blastocysts were fixed in 3% paraformaldehyde/0.5% glutaraldehyde in PBS, rinsed in PBS containing 0.1% Triton X-100 (PBS-TR), exposed to 10 µg/ml proteinase K in PBS-TR and washed in 2 mg/ml glycine in PBS-TR before refixation in 4% paraformaldehyde/0.2% glutaraldehyde in PBS. The blastocysts were then incubated in 0.1% sodium borohydride and prehybridized in a solution described elsewhere (MacPhee et al., 1994). The embryos were hybridized in the same solution containing the probes ISHB+ISHD or ISHA+ISHC at 0.5 µM of each oligonucleotide for 34 hours at 50°C with agitation. Post-hybridization consisted of successive washes in 300 mM NaCl, 1 mM EDTA, 10 mM Pipes and 1% SDS; in 50 mM NaCl, 1 mM EDTA, 10 mM Pipes and 0.1% SDS; in 500 mM NaCl, 10 mM Pipes and 0.1% Triton X-100 before transfer into the latter buffer with 100 µg/ml ribonuclease A and 100 U/ml ribonuclease T1. Post-hybridization was continued in 300 mM NaCl, 1 mM EDTA, 10 mM Pipes, 50% formamide and 1% SDS; in 150 mM NaCl, 1 mM EDTA, 10 mM Pipes, 50% formamide and 0.1% Triton X-100 and in 500 mM NaCl with 0.1% Triton X-100. Following a rinse in PBS supplemented with 0.5% Tween-20 (PBS-T), the embryos were treated in 0.3% hydrogen peroxide in PBS-T, incubated in 0.1% sodium citrate and 0.1% Triton X-100 and then exposed to a streptavidin-biotin-peroxidase complex. The embryos were prestained with 25 µg/ml of HO in PBS-T and transferred into a solution of diaminobenzamide and nickel chloride. Exposure of the embryos to u.v. light allowed to visualize intact and fragmented nuclei whereas visible light allowed for the detection of clusterin mRNA-positive cells.

Statistical analysis

Except for the data in Table 1, which were compared by either Student's *t*-test or Mann-Whitney U-test, differences between control values and experimental values were compared by one-way analysis of variance coupled with Scheffe's F-test. Except in Fig. 9, the data are given as mean±s.e.m. For each set of experiments, blastocysts from 4 to 8 females (normal or diabetic) were pooled before either direct observation or assignment to different incubation groups. Inter-litter variations, if any, have been previously found to be neutralized by this randomization process. In addition, the results in Figs 3, 4, 6 and 7 as well as in Table 2 were based on reproducible data collected from at least three independent and 'blind' repeats.

RESULTS

Embryo collection

On both day 5 and day 6, hyperglycemia and ketosis were confirmed in the diabetic group ($P\leq0.05$) (Table 1). Although the average serum TNF-α value in diabetic rats on day 6 was twice the control value, on neither day was the difference statistically significant. On day 5, both the total number of embryos recovered per rat (9.4±0.2 versus 7.7±0.3) and the number of blastocysts per rat (Table 1) were decreased in the diabetic group. No difference was found on day 6, however, probably because the most advanced blastocysts in the control group had already implanted at the time of uterine flushing.

Nuclear fragmentation in blastocysts in vivo

The occurrence of cell death was analyzed by means of a dual staining technique that discriminates between ICM and TE

Table 1. Recovery of rat blastocysts on gestational day 5 and day 6

	Day 5			Day 6		
Parameter	Control	Diabetic	*P*-value	Control	Diabetic	*P*-value
Blood glycemia (mM)	5.8±0.2	23.3±0.5	**	6.6±0.3	24.3±0.5	**
n	83	74		66	61	
Serum β-hydroxybutyrate (μM)	1.7±0.2	3.3±0.4	**	1.2±0.2	1.9±0.2	*
n	19	20		11	11	
Serum TNF-α (U/ml)	323.4	266.7	0.74	262.1	605.3	0.19
Confidence interval	35.1-611.7	132.9-400.4		89.5-434.6	276.6-1487.2	
n	20	20		20	20	
Number of blastocysts per rat	8.2±0.3	4.9±0.3	**	2.4±0.2	2.7±0.4	0.23
n	79	62		56	46	

Data are means ± s.e.m. with *n* representing the number of values. (*) and (**) indicate $P \leq 0.05$ or 0.01 respectively.

nuclei (Fig. 2). When blastocysts from diabetic rats were collected on day 5 and compared to control embryos, a 18.3% deficit in the total cell number was found in the diabetic group ($P \leq 0.01$) (Fig. 3), with about 4/5 of this loss located to the ICM. Closer examination revealed that the percentage of OH-stained fragmented nuclei increased to 8.4±1.7% in the ICM of blastocysts in the diabetic group from 1.1±0.3% in the ICM of control embryos ($P \leq 0.01$). In contrast, the percentage of PI-stained fragmented nuclei, which was below 1.0% in the TE lineage of control blastocysts, was not statistically different in embryos from diabetic rats. Altogether, 48.9% of the blastocysts in the day 5 diabetic group contained at least one disintegrating nucleus versus 18.2% in the control group. These observations were repeated on blastocysts recovered on day 6. Compared with control embryos, blastocysts in the diabetic group contained fewer cells (16.8% loss) on the average ($P \leq 0.01$) (Fig. 3) with the deficiency again predominantly located to the ICM. The nuclear fragmentation index was increased in the ICM of blastocysts from diabetic rats, 7.1±1.1% against the control value of 2.2±0.9% ($P \leq 0.05$), whereas it remained low in TE cells. Exactly 50% of the blastocysts from day 6 diabetic rats had at least one fragmented nucleus, irrespective of its location, versus 31.4% in the control group.

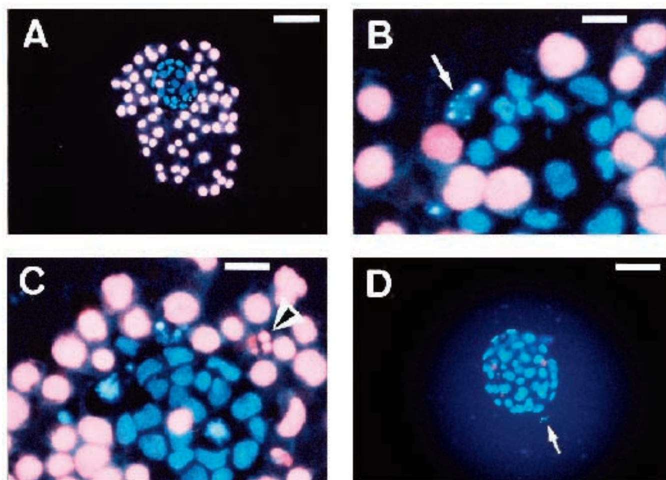

Fig. 2. Nuclear fragmentation in blastocysts. Differential staining with HO and PI was used to distinguish ICM (blue) and TE (pink) nuclei (A). Closer examination revealed the presence of fragmented HO-stained ICM nuclei (B; arrow) and fragmented PI-stained nuclei (C; arrowhead) in some blastocysts. In some blastocysts, expelled cells with HO-stained nuclear fragments were observed in the perivitelline space (D; arrow). Blastocysts were from a control rat (A), from diabetic rats (B, C) or following incubation in high D-glucose (D). The scale bar represents 50 μm in A,D and 10 μm in B,C.

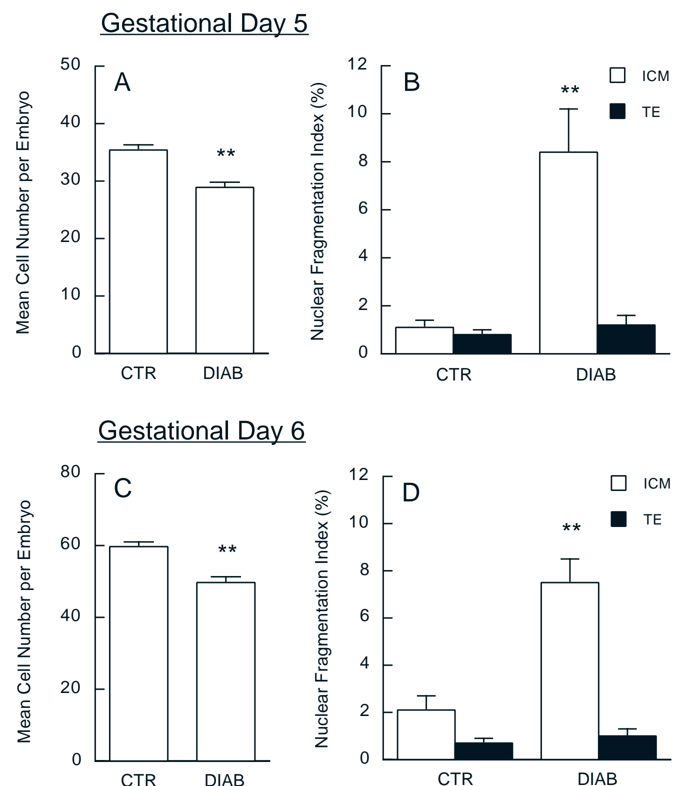

Fig. 3. Nuclear fragmentation in blastocysts exposed to maternal diabetes. Embryos were recovered from control (CTR) and diabetic (DIAB) rats on day 5 (upper panels) or day 6 (lower panels) and counted for the total number of cells per embryo (A,C) and for the frequency of nuclear fragmentation (B,D) in the inner cell mass (ICM) and trophectoderm (TE) lineages. More than 45 embryos were examined in each group on day 5 and more than 30 embryos on day 6. (**) indicates a statistically significant difference from corresponding control value ($P \leq 0.01$).

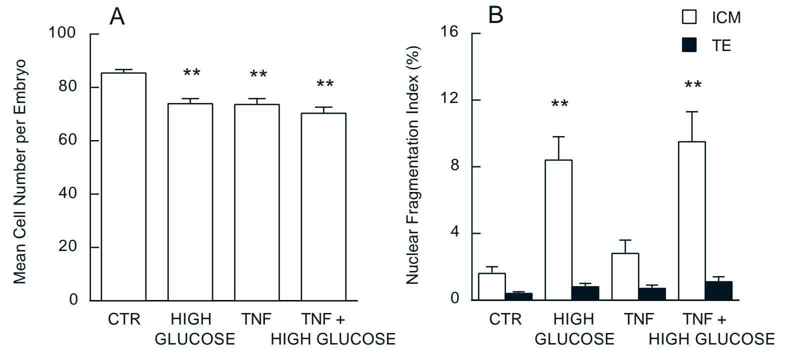

Fig. 4. Nuclear fragmentation in blastocysts exposed to high glucose and TNF-α. Embryos were recovered from normal rats and incubated in control medium (CTR, *n*=55 embryos) or in high D-glucose (HIGH GLUCOSE, *n*=36), TNF-α (TNF, *n*=32) or a combination of high D-glucose and TNF-α (TNF + HIGH GLUCOSE, *n*=32) before examination for the total number of cells per embryo (A) and for the frequency of nuclear fragmentation (B) in the inner cell mass (ICM) and trophectoderm (TE) lineages. (**) indicates a statistically significant difference from corresponding control value ($P \leq 0.01$).

Nuclear fragmentation in blastocysts in vitro

Blastocysts from normal rats were incubated under different conditions. At the initiation of the culture, the mean cell number per blastocyst was 37.6±1.3 with 13.5±0.5 of ICM cells and 24.1±0.9 TE cells. After 24 hours, there was a 2.3-fold increase in the mean cell number per embryo in control culture medium. Compared with control blastocysts, fewer cells were counted in embryos incubated in the presence of 17 mM D-glucose (13.3% loss), 3000 U/ml rat recombinant TNF-α (13.8% loss) or in a combination of high D-glucose and TNF-α (17.7% loss) ($P \leq 0.01$) (Fig. 4). In all these groups, the cell deficiency was more pronounced in the ICM than in the TE. Closer examination showed that the percentage of fragmented ICM nuclei was increased to 8.4±1.4% in high D-glucose versus 1.6±0.4% in control culture medium ($P \leq 0.01$) whereas exposure to TNF-α alone did not induce any significant difference. Combining high D-glucose and TNF-α raised the nuclear fragmentation index in the ICM to 9.5±1.8% ($P \leq 0.01$). None of the treatments tested increased nuclear fragmentation in the TE. At the start of the incubation period, average frequencies of disintegrating nuclei were below 1.0% in both ICM and TE lineages. In additional experiments, blastocysts were incubated in control medium or in high D-glucose or L-glucose for 24 hours and directly transferred into a solution of HO and PI. The incidence of HO-stained fragmented nuclei remained below 0.5% in the peripheral TE layer of all the blastocysts examined. The frequencies of PI-stained intact and fragmented nuclei remained also at a very low level (0.1%). Omission of enzymatic digestion allowed for the observation of extruded cells in the perivitelline space of about 15% of the blastocysts exposed to high D-glucose (Fig. 2). Most of these cells contained HO-stained nuclear fragments.

TUNEL-positive nuclei in blastocysts in vivo

The occurrence of cell death was further analyzed using a technique that allowed for the simultaneous detection of fragmented nuclei in u.v. light and nuclei with abundant free DNA 3′-OH ends (TUNEL-positive nuclei) in visible light (Fig. 5). A comparison between blastocysts from control and diabetic rats on day 5 confirmed that the mean total cell number was decreased in the diabetic group ($P \leq 0.01$) (Fig. 6). In these blastocysts, the F-index and T-index were both increased ($P \leq 0.01$) whereas the presence of nuclei combining signs of fragmentation with TUNEL-staining was not detected (below 0.1%). Only 21.4% of the blastocysts collected from diabetic rats showed no signs of cell death against 73.2% in the control group. The frequency of nuclei in metaphase was not statistically different in embryos from the control and diabetic groups (1.5±0.3% versus 1.2±0.3%). Close examination of 12 blastocysts from diabetic rats in which the blastocoele was clearly delineated showed that 82% of the TUNEL-positive nuclei detected in these embryos were localized in the ICM region. Blastocysts recovered on day 6 were examined using the same technique. All the three nuclear labeling indexes were increased in blastocysts in the diabetic group ($P \leq 0.01$) (Fig. 6). Only 13.2% of the blastocysts from diabetic rats were without signs of nuclear damage, relative to 42.2% in the control group. The percentage of nuclei in metaphase was low (≤0.3%) and

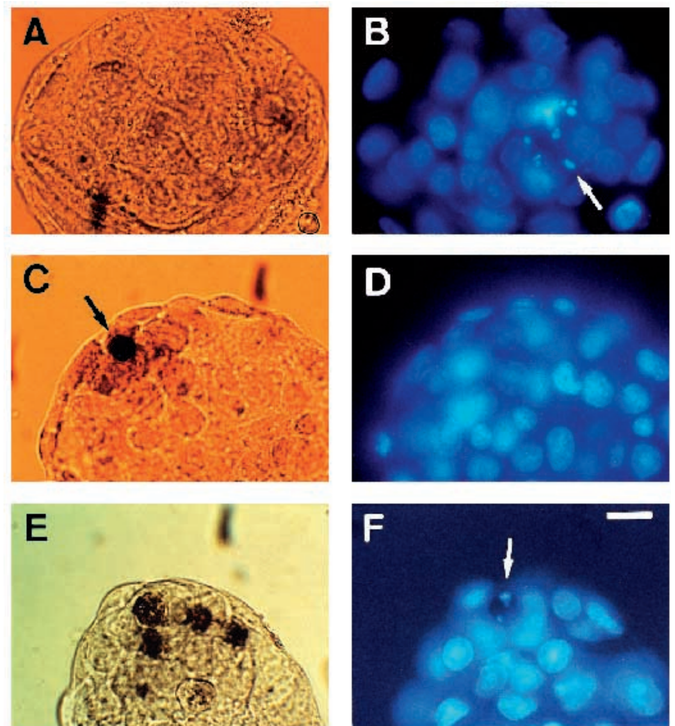

Fig. 5. TUNEL staining in blastocysts. HO staining coupled with TUNEL staining were used to visualize different types of nuclear degradation. Damaged nuclei were classified in three categories according to their labeling pattern: HO-stained fragmented nucleus without TUNEL signal (F-labeling) (A,B; arrow), HO-stained intact nucleus with TUNEL signal (T-labeling) (C; arrow, D) or HO-stained fragmented nucleus with TUNEL signal (F+T labeling) (E,F; arrow). Blastocysts shown were from control (A,B and C,D) and diabetic (E,F) rats. The scale bar in F represents 15 μm.

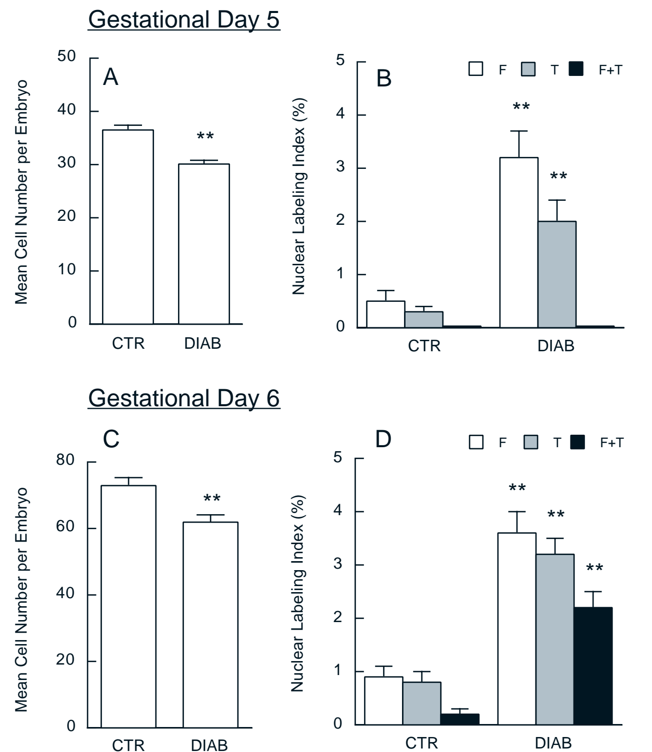

Fig. 6. TUNEL staining in blastocysts exposed to maternal diabetes. Embryos were recovered from control (CTR) and diabetic (DIAB) rats on day 5 (upper panels) or day 6 (lower panels) and counted for the total number of cells per embryo (A,C) and for the frequencies of fragmented nuclei (F), TUNEL-positive nuclei (T) and nuclei displaying both nuclear labels (F+T). More than 40 embryos were examined in each group on day 5 and more than 30 embryos on day 6. (**) indicates statistically significant difference from corresponding control value ($P \leq 0.01$).

identical in the two groups. Because the blastocoele had collapsed in almost all the blastocysts recovered on day 6, it was more difficult to confirm that TUNEL-positive nuclei were still predominantly located in the ICM at that later stage.

TUNEL-positive nuclei in blastocysts in vitro

Normal blastocysts cultured in the presence of either 17 mM D-glucose, 3000 U/ml TNF-α or high D-glucose and TNF-α combined for 24 hours contained fewer cells than blastocysts in control medium ($P \leq 0.05$) (Fig. 7). Compared with control embryos, F-index and T-index were both increased in embryos exposed to high D-glucose ($P \leq 0.01$) although there was no difference in the F+T index. Both T-index and F+T-index, but not nuclear fragmentation alone, were increased in embryos exposed to TNF-α ($P \leq 0.05$). Exposure to high D-glucose and TNF-α simultaneously resulted in an increase in all three nuclear labeling indexes ($P \leq 0.05$). The percentage of blastocysts showing no signs of cell death was 12.5% in the control group versus 12.1% in high D-glucose, 2.9% in TNF-α and 2.3% in high D-glucose combined with TNF-α. The frequency of metaphasic nuclei (≤1.0%) was not influenced by the culture conditions. Careful examination of 19 blastocysts exposed to high D-glucose and 18 blastocysts treated with TNF-α in which the limits of the blastocoele were clearly visible showed that, respectively, 81% and 73% of the TUNEL-positive nuclei detected in these embryos were localized in the ICM region. In 16 control blastocysts, 77% of the TUNEL-positive nuclei were found in the ICM. Addition of 11 mM L-glucose to the control medium did not influence the mean total number of cells per embryo nor the percentages of nuclei showing signs of fragmentation and/or TUNEL-staining (Fig. 7). Preincubation of a culture medium containing 3000 U/ml TNF-α with a 100-fold molar excess of neutralizing rabbit anti-rat TNF-α antibody before embryo culture prevented the impact of the cytokine on the mean number of cells and on the nuclear labeling indexes. When an equivalent molar excess of normal rabbit IgG was substituted for anti-TNF-α antibody, the mean total number of cells per embryo as well as the average T-index

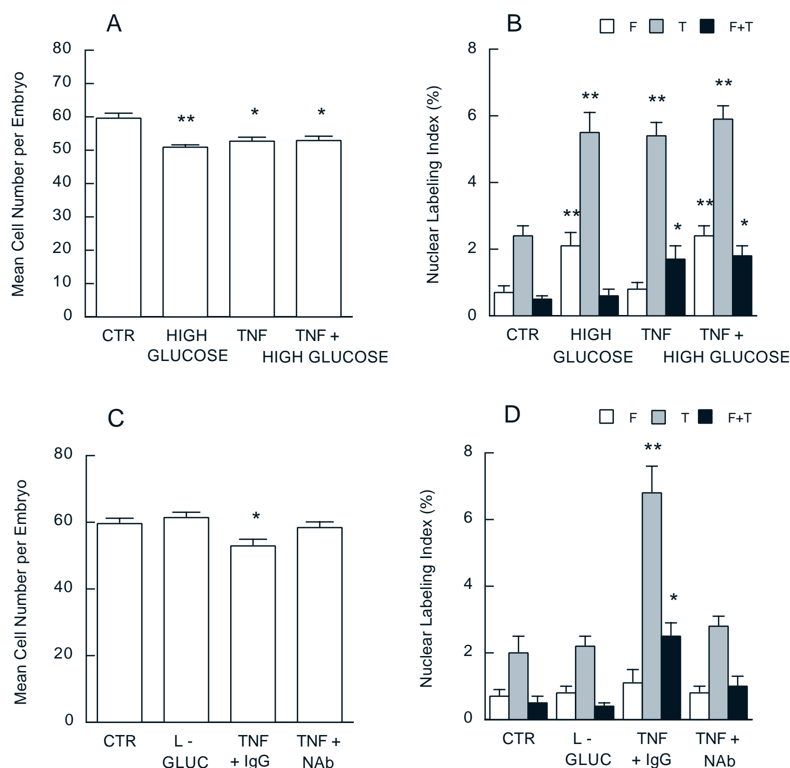

Fig. 7. TUNEL staining in blastocysts exposed to high glucose and TNF-α. Embryos were recovered from normal rats and incubated in control medium (CTR, n=48 embryos) or in high D-glucose (HIGH GLUCOSE, n=33), TNF-α (TNF, n=35) or a combination of high D-glucose and TNF-α (TNF+HIGH GLUCOSE, n=42) (upper panels) or in 6 mM D-glucose with 11 mM L-glucose (L-GLUC, n=23), TNF-α plus normal IgG (TNF+IgG, n=27) or TNF-α neutralized with blocking anti-TNF-α antibodies (TNF+NAb, n=29) before examination for the total number of cells per embryo (A,C) and for the frequencies of fragmented nuclei (F), TUNEL-positive nuclei (F+T) and nuclei displaying both nuclear labels (F+T) (B,D). (*) and (**) indicate statistically significant differences from corresponding control values ($P \leq 0.05$ and $P \leq 0.01$, respectively).

and F+T-index were altered to the same extent as these values were in the presence of TNF-α alone ($P \leq 0.05$). In a series of negative control reactions, blastocysts were incubated in 17 mM D-glucose for 24 hours and subjected to the TUNEL-staining procedure without terminal transferase. Nine embryos, with a mean total number of cells of 49.1±2.3 and an average frequency of fragmented nuclei of 5.7±0.6%, were found completely free of TUNEL staining. In a series of positive control reactions, blastocysts were cultured in control medium for 24 hours and then treated with deoxyribonuclease I for 2 hours before TUNEL staining. Examination of 5 blastocysts showed that the proportion of TUNEL-positive nuclei was increased to 49.3±5.9%. The high embryotoxicity of the enzyme buffer made it difficult to test whether a longer exposure time would result in an even higher T-index.

Clusterin mRNA expression in blastocysts

Amplification of cDNA preparations from blastocysts recovered from both control and diabetic rats with clusterin primers SP1 and SP2 generated an amplicon of the expected size (564 bp) (Fig. 8). Positive control amplifications performed on cDNAs from either rat ovaries or limb buds obtained from day 15 fetuses (a developing organ rich in clusterin mRNA; Buttyan et al., 1989) produced the same amplicon. In contrast, amplification of cDNA from morulae was negative. When day 5 blastocysts from control or diabetic rats were compared by means of a quantitative RT-PCR method, the average level of clusterin mRNA was 57.7±9.1 arbitrary units in the control group versus 101.1±5.5 arbitrary units in the diabetic group ($P \leq 0.01$) (Fig. 9). In a second series of experiments, the presence of clusterin transcripts was analyzed in normal blastocysts incubated for 24 hours in either 17 mM D-glucose, 3000 U/ml TNF-α or a combination of high D-glucose and TNF-α. Compared with embryos maintained in control medium (50.6±2.5 arbitrary units), the average level of clusterin mRNA was increased to 136.5±11.4 arbitrary units in blastocysts exposed to high D-glucose alone and 158.2±14.1 arbitrary units in high D-glucose with TNF-α ($P \leq 0.01$) (Fig. 9). Exposure to TNF-α alone did not influence clusterin mRNA expression. In addition, there was no significant difference between blastocysts treated with high D-glucose alone or with high D-glucose and TNF-α combined.

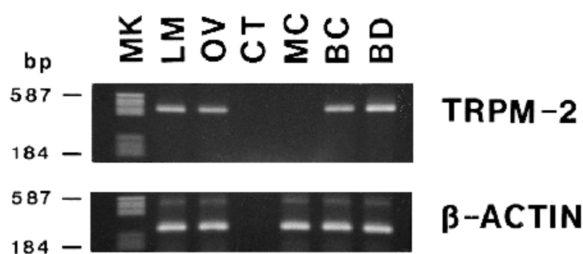

Fig. 8. Detection of clusterin transcripts in blastocysts by RT-PCR. Total cDNA from rat fetal limb (LM), adult ovary (OV), morulae (MC) and blastocysts (BC) from control rats as well as blastocysts from diabetic rats (BD) were tested for clusterin/TRPM-2 mRNA expression using specific primers expected to generate a 564 bp-long amplicon (upper panel). The same cDNAs were amplified for β-actin in control reactions (lower panel). DNA size markers (MK) were run in the first gel lane.

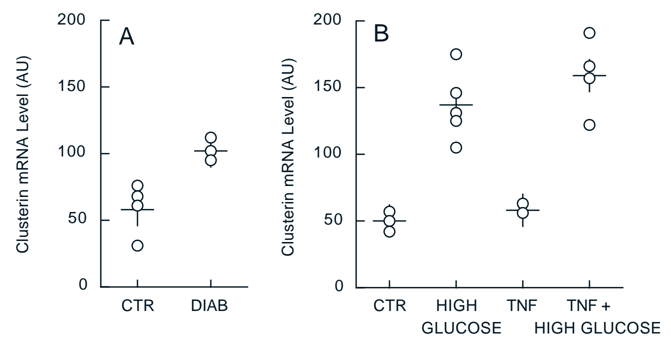

Fig. 9. Clusterin mRNA expression in blastocysts exposed to maternal diabetes or to high glucose and TNF-α. Total cDNAs from blastocysts collected from control (CTR, n=4 pools) and diabetic (DIAB, n=3) rats on day 5 (A) or from normal rats and incubated in control medium (CTR, n=3 pools), high D-glucose (HIGH GLUCOSE, n=5), TNF-α (TNF, n=2) or a combination of high D-glucose and TNF-α (TNF+HIGH GLUCOSE, n=4) were analyzed for clusterin expression by quantitative RT-PCR. Quantitative values for individual blastocyst pools are represented (open circles) and the average expression level in each group is indicated by a horizontal bar.

Localization of clusterin mRNA expression

Control and diabetic blastocysts were collected on day 5 and maintained overnight in normal culture medium before being analyzed by ISH. Although 81% of the blastocysts from diabetic rats were found to contain at least one clusterin mRNA-positive cell, the frequency of these cells per embryo was low (1.6±0.2 such cell per embryo). Simultaneous staining with HO demonstrated that, in 46% of the clusterin mRNA-positive cells, the ISH labeling in the cytoplasm correlated with nuclear fragmentation (Fig. 10). The same pattern was found in blastocysts incubated for 24 hours in high D-glucose, with 92% of the embryos containing at least one clusterin-expressing cell, an average of 1.5±0.2 such cell per embryo and 48% of these cells displaying signs of nuclear disintegration. In these two blastocyst groups, the incidence of clusterin-expressing cells was higher than in control embryos ($P \leq 0.01$) (Table 2). In contrast, ISH analysis of blastocysts exposed to TNF-α did not detect an increase in the proportion of clusterin-expressing cells. About half the TNF-α-treated blastocysts contained at least one clusterin mRNA-positive cell, with an average of 0.6±0.2 such cell per embryo, and 49% of these cells also displayed signs of nuclear fragmentation. Overall, the ISH

Table 2. In situ hybridization for clusterin mRNA expression in blastocysts

Parameter	Control group	Diabetic group	Incubation in high glucose	Incubation in TNF-α
Total cell number	59.2±1.9	42.1±1.5**	51.8±1.4**	50.4±1.9**
Nuclear fragmentation index (%)	1.2±0.3	6.9±0.7**	4.7±0.7**	2.5±0.4
Clusterin-positive cell frequency (%)	0.6±0.2	3.9±0.6**	2.9±0.4**	1.4±0.4
n	18	21	24	21

Data are means ± s.e.m. with n representing the number of values. (**) indicates $P \leq 0.01$.

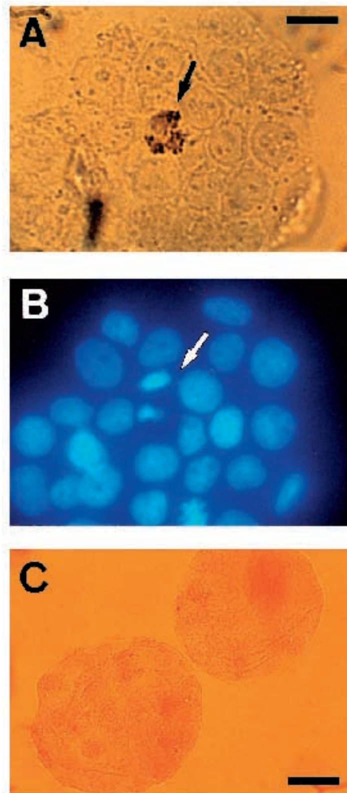

Fig. 10. Localization of cells expressing clusterin transcripts by ISH. HO staining coupled with ISH were used to identify the cells overexpressing clusterin in blastocysts. In about half the cells found to contain high levels of clusterin transcripts, the nucleus was in fragmentation, as exemplified in the blastocyst from a diabetic rat presented here (A,B; arrows). Blastocysts exposed to high D-glucose and hybridized to the control ISH probes did not show any ISH signal (C). The scale bar represents 10 μm in A,B and 30 μm in C.

data were consistent with the RT-PCR results. Experiments on 20 blastocysts exposed to high D-glucose and hybridized to the control sense probes demonstrated the complete lack of non-specific signal (Fig. 10).

DISCUSSION

Cell death in blastocysts exposed to diabetes in utero

Exposing blastocysts to the DNA fluorochromes HO and PI following TE immunolysis showed that a high proportion of cells identified as ICM cells showed signs of nuclear fragmentation in blastocysts from day 5 and day 6 diabetic rats. The fact that these dying cells were stained only with HO indicated that they were still delimited by intact membrane boundaries. Although ICM cells in advanced degradation stages would take PI when membrane integrity is lost and risk being miscounted as TE cells, this possibility is considered as unlikely because the proportion of cells with PI-stained nuclear fragments was equally low in both embryo groups. Because cells in very early stages of the suicidal process may already have subtle chromatin alterations before nuclear fragmentation occurs, blastocysts were also examined using the TUNEL method coupled with HO staining. This technique revealed the presence in blastocysts from day 5 diabetic rats of a population of dying cells distinct from those identified on the basis of HO-stained nuclear fragments. This distinction was confirmed when blastocysts were investigated on day 6, with the additional observation of yet another population of dying cells whose nuclei were displaying the two markers simultaneously (HO-stained DNA particles within a TUNEL-positive nucleus). The observation that not all TUNEL-positive nuclei were in the process of disintegration, a phenomenon also recently described in mouse blastocysts cultured under suboptimal conditions (Brison and Schultz, 1997), is still unexplained, mostly because it is unclear whether these two features form a directly coupled sequence of nuclear events. Uncertainties as to the nature of the initial event(s) leading to the DNA damages identified by the TUNEL technique as well as the duration of fragmentation and TUNEL detection in cells undergoing cell death also complicate the interpretation (Gold et al., 1994; Didenko and Hornsby, 1996). Although no attempt was made to combine TUNEL staining with ICM-TE differential staining, it was clear that most of the TUNEL-positive and/or fragmented nuclei were in cells situated within the ICM region. To further describe the occurrence of cell death in embryos, the mRNA expression of clusterin was quantitated by RT-PCR and localized by ISH in blastocysts from diabetic rats. Production of clusterin has been associated with cell death in several systems (Buttyan et al., 1989). One of its many proposed functions is the capture and neutralization of cellular debris resulting from the degradation of dying cells (Koch-Brandt and Morgans, 1996), a function clusterin might perform for instance during the remodeling process of blastocyst implantation (Brown et al., 1996). According to the present study, control rat embryos were found to start expressing clusterin mRNA at the blastocyst stage. Previous studies have demonstrated that cells in the TE lineage selectively expressed the gp330 clusterin receptor in the rat (Sahali et al., 1993) as well as in the mouse (Guet-Hallonet et al., 1994) blastocyst, findings that together with our data support the concept that a clusterin / gp330-based system may participate in the clearance of dead cell fragments inside embryos. Compared with control blastocysts, the relative amount of clusterin transcripts was increased two-fold in blastocysts from diabetic rats on day 5. Up-regulated clusterin expression was confined to a very limited number of cells, many of them displaying signs of nuclear fragmentation. Thus analysis of clusterin expression on an individual basis revealed a close correlation with the process of cell death in blastocysts. Controversial results exist about whether cells accounting for an elevation in clusterin expression within a site of cellular elimination are those undergoing cell death or neighboring cells destined to survive. Studies aimed at identifying clusterin-expressing cells and dying cells in the human thymus (French et al., 1992) and in various lympho-hematopoietic cell lines (French et al., 1994) support the idea that only viable cells produce clusterin. In contrast, simultaneous display of nuclear degradation and clusterin transcription has been reported in individual glandular epithelial cells in the mouse uterus (Ahuja et al., 1994). A reconciling view would be that, in certain situations, such as in blastocysts, dying cells may engage into the altruistic process of up-regulating their synthesis of clusterin in order to facilitate the removal of their own debris by surrounding clusterin receptor-expressing cells.

Cell death in blastocysts exposed to high glucose

Previous reports have shown that hyperglycemia per se is directly toxic to certain cell populations, such as human endothelial cells (Lorenzi et al., 1985), and increases the expression of several markers of active cell death (Baumgartner-Parzer et al., 1995). Rat blastocysts incubated in high glucose were found to contain fewer cells than control embryos, with the cell

loss occurring mainly at the expense of the ICM. High glucose also induced an five-fold increase of the proportion of ICM cells displaying nuclear fragmentation within intact cell membranes. When blastocysts were subjected to the TUNEL technique paired with HO staining, both the F-labeling and T-labeling indexes were found increased following culture in high glucose. Substituting non-metabolizable L-glucose for D-glucose in cultures demonstrated the specificity of the active form of the nutrient. Additional experiments aimed at assessing the expression of clusterin showed an increase in the relative abundance of transcripts in blastocysts incubated in high glucose. About half the cells expressing high levels of clusterin transcripts also contain a fragmented nucleus, indicating again a close relationship between individual cell death and clusterin up-regulation. The mechanism by which moribund cells may be cleared from within a blastocyst is not known. In contrast with most situations where resident macrophages could rapidly phagocytose cell fragments, the phagocytotic potential of embryonic cells is probably limited (El-Shershaby and Hinchliffe, 1974). Embryonic dead cells that can not be phagocytosed by adjacent viable cells would therefore undergo in situ degeneration or expulsion into the perivitelline space. Our observations revealed the presence of such extruded cells (containing a fragmented nucleus within an intact cell membrane) on the outer edge of several blastocysts. This was consistent with a previous description of dead cell extrusion in blastocysts from diabetic BB/E rats (Lea et al., 1996).

Cell death in blastocysts exposed to TNF-α

Previous studies have shown that the synthesis of TNF-α is up-regulated in the uterus of the pregnant diabetic rat (Pampfer et al., 1995a) as well as in primary cultures of rat uterine cells incubated in high glucose (Pampfer et al., 1997). These data suggest that blastocysts developing in diabetic rats may be exposed to abnormally high concentrations of TNF-α. Cell death induction is clearly one of the most prominent biological activities exerted by TNF-α (Nagata, 1997) and several reproductive cell types, such as rat trophoblasts (Hunt et al., 1989; Roby et al., 1994), have been shown to be sensitive to the cytotoxic action of the cytokine. When rat recombinant TNF-α was tested at a concentration about 5 times higher than the circulating concentration of TNF-α in day 6 diabetic rats, no significant increase was observed in the incidence of nuclear fragmentation in blastocysts. These data confirmed earlier results showing that mouse recombinant TNF-α could induce a decrease in the total number of cells per rat blastocyst without increasing the rate of nuclear fragmentation in these embryos (Pampfer et al., 1994b) and were consistent with other observations that DNA fragmentation is not always involved in the cytotoxic process triggered by the cytokine (Mirkina et al., 1996). This lack of nuclear fragmentation was tentatively explained by the fact that rat blastocyst cells, like several other cell types similarly resistant to TNF-α-induced DNA fragmentation (Higuchi and Aggarwal, 1994), express only the p60 isoform of TNF-α receptor (Pampfer et al., 1995b). In contrast, the cytokine was found to double the T-index and to triple the F+T-index, thus confirming the ability of the cytokine to up-regulate the expression of some markers of cell death in embryos. The extent of the increase in T-index in rat blastocysts was consistent with the recently described 2.5-fold increase in the proportion of TUNEL-positive cells among TNF-α-treated human trophoblasts (Yui et al., 1994). Because of previous indications that TNF-α can up-regulate clusterin expression in mouse fibrosarcoma cells (Kyprianou et al., 1991) and in human prostatic cells (Sensibar et al., 1995), it was expected that TNF-α would increase the relative mRNA level of clusterin in rat embryos. The data showed that the cytokine did not influence the steady-state transcription level of clusterin in blastocysts.

Cell death in blastocysts exposed to high glucose and TNF-α

Co-exposure of the blastocysts to high glucose and TNF-α was found to recapitulate the different effects observed in embryos recovered from diabetic rats on day 6. Increases in all three nuclear labeling indexes were detected after treatment with high glucose and TNF-α. Clusterin mRNA expression was also stimulated following this combined exposure. No additive effect was seen in the frequency of TUNEL-positive nuclei, a cell death parameter that was up-regulated by both glucose and TNF-α, suggesting that the cell death mechanisms triggered by the two effectors might converge at some limiting point.

CONCLUSION

Overall, the data demonstrate that several markers of active cell death are increased in blastocysts exposed to maternal diabetes. Excessive cell death occurred predominantly in the ICM. Incubation of normal blastocysts in high D-glucose, and to a lesser extent in TNF-α, also increased the expression of these markers. Because cell death is constitutively detected at a very low level in control blastocysts, it is concluded that the deleterious impact of maternal diabetes on embryo development (Pampfer and De Hertogh, 1996) may be mediated by the inappropriate up-regulation of this regular elimination process. The observation of clusterin overexpression in blastocysts from diabetic rats indicates that, in addition to suffering from a cell deficiency in their vital ICM lineage, these embryos may be affected by subtle disruptions in the expression pattern of critical developmental genes.

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