Expression and Role of Bcl-2 in Rat Blastocysts Exposed to High D-Glucose

Serge Pampfer, Sabine Cordi, Ivo Vanderheyden, Patrick Van Der Smissen, Pierre J. Courtoy, Anne Van Cauwenberge, Henri Alexandre, Isabelle Donnay, and René De Hertogh

Bcl-2 mRNA expression was detected in rat blastocysts by in situ hybridization. The distribution of mRNA expression was rather heterogenous, with ~2% of high-expressing cells. In vitro exposure to 28 mmol/l D-glucose for 24 h resulted in a significant increase in the proportion of these cells compared with control embryos in either 6 mmol/l D-glucose or 28 mmol/l D+L-glucose. Heterogeneity in the expression of Bcl-2 was also observed at the protein level by immunocytochemistry. Exposure to 28 mmol/l D-glucose significantly increased the incidence of chromatin degradation (karyolysis) and nuclear fragmentation (karyorhexis), two nuclear markers of apoptosis in rat blastocysts. When two different antisense oligodeoxynucleotides designed to block Bcl-2 expression were added to 28 mmol/l D-glucose, the incidence of karyolysis (but not karyorhexis) was increased compared with embryos in 28 mmol/l D-glucose alone. These data suggest that Bcl-2 is involved in the protective response against the induction of karyolysis in blastocysts on their exposure to high concentrations of D-glucose in vitro, whereas karyorhexis appears to result from the activation of an intracellular pathway that is independent of Bcl-2. *Diabetes* 50:143–149, 2001

In addition to playing a fundamental role in adult homeostasis, apoptosis is known to be instrumental in the formation and maturation of many developmental systems (1). Noticeable developmental cell death occurs at the blastocyst stage (2–3) when embryonic cells engage in a subtle process of differentiation that leads to the emergence of two distinct cell lineages, the fetal precursor inner cell mass (ICM) and the placental precursor trophectoderm (TE). However, under normal conditions, the proportion of cells showing signs of self-destruction remains limited, and this process is predominantly located to the ICM lineage (4). In contrast to apoptotic events that are programmed later in the course of development, the restricted wave of cell death that is detected at the blastocyst stage does not seem to serve an obvious morphogenetic purpose. The proposed explanation for its occurrence is that this process may allow for the efficient elimination of abnormal or redundant ICM cells before gastrulation is initiated.

Previous studies have shown abnormally high incidences of nuclear fragmentation (karyorhexis) (5–6) and chromatin degradation (karyolysis) (7) in ICM cells of blastocysts recovered from insulin-dependent diabetic rats, suggesting that excess ICM cell death may be a factor contributing to the severe embryopathy that has been associated with preconceptional maternal diabetes (8–9). Incubating blastocysts from normal rats in high concentrations of D-glucose was also found to stimulate karyorhexis (10) and karyolysis (7) in the ICM cell lineage of rat blastocysts, in support of the hypothesis that hyperglycemia plays a direct role in the induction of the cellular alterations observed in utero. Observations made on blastocysts from diabetic mice and on mouse embryos after in vitro culture in high concentrations of D-glucose (11) largely confirmed the data reported on rat embryos.

There is evidence that several genes of the Bcl-2 and caspase families, which are known intracellular regulative and executive components of the apoptotic cascade, are already expressed at the blastocyst stage (12–13). Treating mouse blastocysts with a combination of protein synthesis and protein kinase inhibitor to desensitize them from survival signals was found to induce massive apoptosis (14–15), pointing towards the existence of a fully operational cell death machinery at that developmental stage. Recent observations also showed that expression of Bax, a proapoptotic member of the Bcl-2 family, was increased in blastocysts from diabetic mice (11) and that expression of clusterin, an apolipoprotein involved in the scavenging of apoptotic cellular debris, was increased in blastocysts from diabetic rats (7). These observations were confirmed in vitro when embryos from normal females were incubated in high concentrations of glucose (7–11) and suggest that exposing preimplantation embryos to hyperglycemia may directly dysregulate the expression of several genes whose products are involved in the induction or execution of apoptosis.

In the present work, we examined whether the mRNA and protein expression of Bcl-2, the antiapoptotic founding member of the eponymous gene family, was modified in rat blastocysts upon their exposure in vitro to 28 mmol/l D-glucose (vs. control cultures in 6 mmol/l). In addition, we have analyzed the consequence of blocking Bcl-2 synthesis in these embryos when exposed to high D-glucose.

From the Physiology of Human Reproduction Research Unit (S.P., S.C., I.V., R.D.H.), Université Catholique de Louvain, Brussels; the Cell Biology Unit (P.V.D.S., P.J.C.), Christian de Duve Institute of Cellular Pathology, Brussels, Belgium; the Division of Biology and Embryology (A.V.C., H.A.), Université de Mons-Hainaut, Mons and the Veterinary Unit (I.D.), Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

Address correspondence and reprint requests to Serge Pampfer, PhD, OBST 5330 Research Unit, University of Louvain Medical School, 53 Avenue Mounier, 1200 Brussels, Belgium. E-mail: pampfer@obst.ucl.ac.be.

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AS, antisense; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; ICM, inner cell mass; PBS, phosphate-buffered saline; PBS-T, PBS with Tween; PBS-TR, PBS containing Triton; RT-PCR, reverse transcriptase–polymerase chain reaction; SN, control sense; TE, trophectoderm; TM, transmembrane; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.

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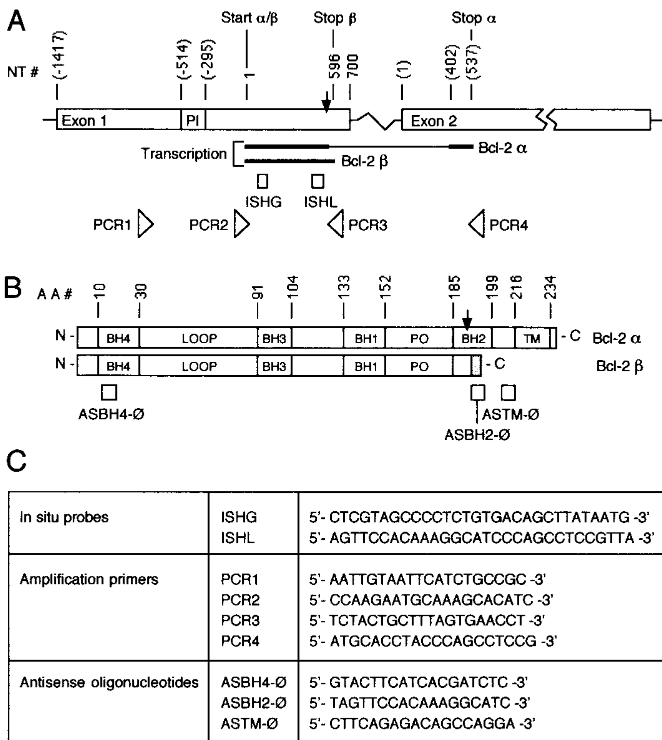

In situ probes	ISHG ISHL	5'- CTCGTAGCCCCTCTGTGACAGCTTATAATG -3' 5'- AGTTCCACAAAGGCATCCCAGCCTCCGTTA -3'
Amplification primers	PCR1 PCR2 PCR3 PCR4	5'- AATTGTAATTCATCTGCCGC -3' 5'- CCAAGAATGCAAAGCACATC -3' 5'- TCTACTGCTTTAGTGAACCT -3' 5'- ATGCACCTACCCAGCCTCCG -3'
Antisense oligonucleotides	ASBH4-Ø ASBH2-Ø ASTM-Ø	5'- GTACTTCATCACGATCTC -3' 5'- TAGTTCCACAAAGGCATC -3' 5'- CTTCAGAGACAGCCAGGA -3'

FIG. 1. Position and sequence of the different Bcl-2 probes, primers, and antisense oligodeoxynucleotides. *A*: Organization of the Bcl-2 gene with the position of the first nucleotide (NT#) in the starting ATG codon (common to Bcl-2α and Bcl-2β) in the first exon numbered as position 1. Numbering in the second exon restarts at the first nucleotide of that exon. The location of the conserved putative intron in exon 1 is indicated. The alternative mRNA splicing site in exon 1 is indicated by (▾) and defines the two possible Bcl-2 transcripts, which stop in either exon 2 (Bcl-2α) or exon 1 (Bcl-2β). The positions of the in situ hybridization probes (□ labeled ISHG and ISHL) and those of the amplification primers (▷ labeled PCR1, PCR2, PCR3, and PCR4) are indicated relative to the Bcl-2 gene sequence. *B*: Presentation of the different domains of the Bcl-2 protein with the first N-terminal amino acid (AA#) numbered as position 1. The seven functional domains identified in the Bcl-2 protein are BH4, phosphorylation loop (LOOP), BH3, BH1, pore-formation (PO), BH2, and transmembrane (TM). The site of the divergence between Bcl-2α and Bcl-2β (midway into the BH2 domain), which is secondary to the alternative splicing decision, is indicated by (▾). The Bcl-2β–specific C-terminus has no known function (■). The positions of the antisense oligodeoxynucleotides (□ labeled ASBH4-Ø, ASBH2-Ø, and ASTM-Ø) are indicated relative to the different domains of the Bcl-2 protein. *C*: Nucleotide sequences of the in situ hybridization probes, amplification primers, and antisense oligodeoxynucleotides described in *A* and *B*.

RESEARCH DESIGN AND METHODS

Embryo collection and culture. Early blastocysts were recovered from the uterine horns of Wistar rats and examined immediately after collection or after culture for 24 h at 37°C in Ham's F-10 medium (Life Technologies) complemented with 1 mmol/l glutamine, 0.1% bovine serum albumin (BSA), 100 U/ml penicillin, and 100 µg/ml streptomycin. The incubation medium was supplemented with either 6 mmol/l D-glucose, 28 mmol/l D-glucose, or a combination of 6 mmol/l D-glucose and 22 mmol/l L-glucose. In some experiments, blastocysts were maintained for 24 h in 17 mmol/l D-glucose.

In situ hybridization. Blastocysts were fixed in 3% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS), incubated in 10 µg/ml proteinase K in PBS containing 0.1% Triton X-100 (PBS-TR), and washed in 2 mg/ml of PBS-TR before refixation in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS. The embryos were treated with 0.1% sodium borohydride, prehybridized, and then exposed to a combination of two 5′-end biotinylated oligodeoxynucleotides (custom synthesized by Life Technologies). These probes were complementary to two distinct regions that were common to both rat Bcl-2α and Bcl-2β (Fig. 1). These two antisense oligodeoxynucleotides were code named ISHG and ISHL and were used at the final concentration of 0.5 µmol/l. Negative control reactions were performed with a combination of corresponding reverse complementary oligodeoxynucleotides (sense oligodeoxynucleotides, code named ISHF and ISHK). After posthybridization and washing in PBS with 0.5% Tween-20 (PBS-T0.5), the embryos were blocked in 0.3% hydrogen peroxide in PBS-T0.5, permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate, and incubated with peroxidase-conjugated streptavidin. The embryos were counterstained with 25 µg/ml Hoechst 33258 in PBS-T0.5 and developed in diaminobenzidine and nickel chloride. For each blastocyst, the proportion of cells strongly positive for Bcl-2 mRNA was expressed as a percentage of its total cell number.

Immunocytochemistry. Blastocysts were treated in acidic Tyrode solution, transferred onto concanavalin A–coated coverslips, and centrifuged at 180*g* for 10 min. The embryos were then fixed in 1.7% paraformaldehyde in PBS, permeabilized in 1% Triton X-100 in PBS, and incubated overnight at 4°C in 0.5 µg/ml rabbit anti-human Bcl-2 primary antibody (Santa Cruz Biotechnology) in PBS with 1% Tween-20 (PBS-T1) and 3% BSA. Negative control reactions consisted in either replacing the primary antibody with normal rabbit IgG or omitting the primary antibody from the procedure. The embryos were then treated with 5.5 µg/ml fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit IgG secondary antibody (Sigma Chemicals) in PBS-T1 for 60 min at 37°C, counter-stained in 1 µmol/l Topro-3 iodide (Molecular Probes) in PBS-T1, and mounted before examination on a confocal laser scanning microscope. Some embryos were observed without prior treatment with antibodies to verify the absence of autofluorescence signal.

Reverse transcription and polymerase chain reaction. Total RNA was isolated from blastocysts after lysis in 4 mol/l guanidine thiocyanate with 0.5 mol/l 2-mercaptoethanol and then treated with 10 U DNase I per reaction for 30 min at 37°C in the presence of 40 U RNase inhibitor. After phenol-chloroform-isoamylalcohol extraction, purified RNA was retrotranscribed with 120 pmoles poly(dT)15 primers and 50 U reverse transcriptase (Roche Molecular Biochemicals) per reaction for 45 min at 42°C. Fresh enzyme was added to the reaction before repeating the incubation. Total cDNA was then amplified with 25 pmoles rat Bcl-2–specific primers and 3.5 U Taq-Pwo DNA polymerases (Roche Molecular Biochemicals). Upstream primers code named PCR1 and PCR2 were complementary to sequences in Bcl-2 exon 1 that are common to Bcl-2α and Bcl-2β, whereas downstream primer PCR3 was located in exon 2 and therefore specific for Bcl-2α (Fig. 1). Primer PCR4 was located in the alternatively transcribed end of exon 1 and hence specific for Bcl-2β. Amplicons were visualized by horizontal gel electrophoresis. To verify their sequence, these amplicons were also directly inserted into pCR 2.1 T/A cloning vectors (InVitrogen) and analyzed on both strands using M13 reverse or M13 forward primers. Products of thermal cycle sequencing reactions were analyzed by vertical gel electrophoresis.

Antisense oligodeoxynucleotides. Antisense (AS) oligodeoxynucleotides were designed against different regions (domains BH4, BH2, and transmembrane [TM]) of the rat Bcl-2α transcript and code named accordingly (Fig. 1). Phosphorothioate linkages were incorporated in the last three 3′-end internucleotide phosphodiester bridges (custom-synthesized by Biosource). Public sequence databases were searched repeatedly to confirm the specificity of these oligodeoxynucleotides against Bcl-2. Reverse complementary control sense (SN) oligos were synthesized for each AS oligo. Before studying the effect of the different anti–Bcl-2 antisense oligodeoxynucleotides on blastocysts exposed to high D-glucose, the action of these inhibitors was first tested in a cell-free assay. Synthetic RNA was generated by in vitro transcription of 0.7 µg of a plasmid containing the entire coding sequence of mouse Bcl-2α (mBcl-2α/pcDNAI is from C. Borner, University of Friboug, Switzerland) and translated using an optimized rabbit reticulocyte lysate (Novagen) supplemented or not with 5 U of RNase H in the presence of 40 µCi of (^{35}S)-L-methionine. Sense and antisense oligodeoxynucleotides were added at the concentration of 15 µmol/l before the initiation of the transcription-translation reaction. Protein synthesis was then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. Preliminary embryotoxicity experiments were then performed on blastocysts. Embryos were cultured in increasing concentrations of AS or SN oligos (0–15 µmol/l) for 24 h and then incubated in a hypotonic solution of 0.9% sodium citrate, fixed in acetic acid and ethanol (3:1), and stained in 4% Giemsa before cell number counting. Irrespectively of the oligo tested, no embryotoxicity was detected for concentrations up to 15 µmol/l (data not shown). In a third set of preliminary experiments, blastocysts were incubated for 24 h in 15 µmol/l of sense or antisense oligodeoxynucleotides and then subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with different pairs of primers. PCR2 and PCR3 were used to amplify Bcl-2 mRNA whereas primer sets specifically designed to amplify either rat β-actin (code named ACT1 [5′-ATGGGTCAGAAGGACTCCTA-3′], ACT2 [5′-ACACAGAGTACTTGC GCTCA-3′]) or rat glyceraldehyde 3-phosphate dehydrogenase (code named

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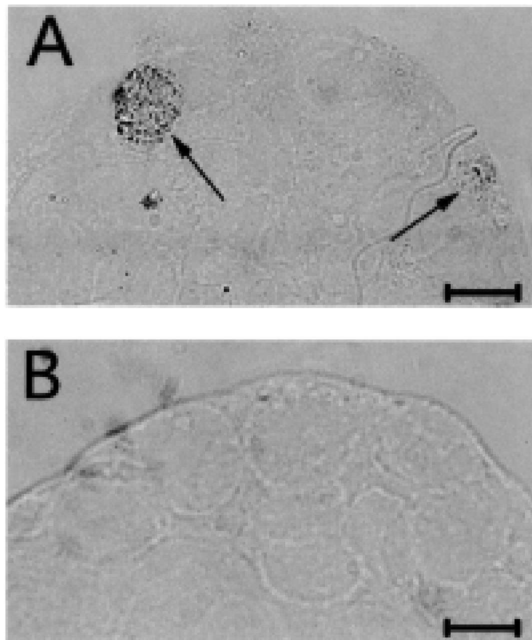

FIG. 2. Bcl-2 mRNA expression in rat blastocysts. *A*: In situ hybridization was performed on a freshly collected blastocyst using a biotinylated antisense probe mixture for Bcl-2. In the small portion of the blastocyst, featured here at high magnification, two strongly Bcl-2$^+$ cells are identified (➤). This embryo is representative of a total of 25 blastocysts that were analyzed for Bcl-2 mRNA expression by in situ hybridization immediately after recovery from the uterine horns. *B*: In situ hybridization using a negative control sense probe mixture for Bcl-2. No specific ISH signal was detected above weak homogenous background. The scale bar represents 10 µm in *A* and *B*.

GPD1 [5′-CCATGGAGAAGGCTGGGG-3′], GDP2 [5′-CAAAGTTGTCATGGATGACC-3′]) were used in positive control reactions.

Detection of karyolysis and karyorhexis. Blastocysts were exposed to 0.4% pronase, fixed in 4% paraformaldehyde in PBS, blocked with 0.3% hydrogen peroxide in methanol, and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. The embryos were then prestained with 25 µg/ml Hoechst 33258 in PBS with 0.5% Tween-20 (PBS-T0.5) and incubated in 50 U/ml of terminal transferase and 15 µmol/l fluorescein-conjugated dUTP (Roche Molecular Biochemicals) for 35 min at 37°C. The addition of terminal transferase was omitted in control negative reactions. After incubation in peroxidase-conjugated sheep antifluorescein antibody, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining was developed in diaminobenzamide and nickel chloride. For each blastocyst, the proportions of cells displaying signs of karyolysis (TUNEL-positive spherical nucleus) and karyorhexis (Hoechst-stained nuclear fragments) were expressed as percentages of the total cell number.

Statistical analysis. Differences between control values and experimental values were compared by one-way analysis of variance coupled with post hoc Scheffe's F-test. The data were given as means ± SE. For each experiment blastocysts from at least 5 females were pooled and randomized before direct observation or assignement to different incubation groups.

RESULTS

Bcl-2 mRNA and protein expression in control embryos. Blastocysts were analyzed by in situ hybridization for the expression of Bcl-2 mRNA using a mixture of antisense ISHG and ISHL probes. In almost every blastocyst that was examined at the time of recovery, only 1–2% of the total number of cells per embryo were strongly positive (Fig. 2). The same proportion of cells with a high ISH signal was found in blastocysts that had been cultured for 24 h in 6 mmol/l D-glucose (see below). Cells with a high Bcl-2 mRNA content did not seem to be preferentially localized in the ICM or TE lineage. Complete absence of staining was found when blastocysts were hybridized to corresponding control ISHF and ISHK sense probes (Fig. 2) or when probes were omitted altogether from the procedure (data not shown).

Blastocysts were examined by immunocytochemistry for the synthesis of Bcl-2. The cell-to-cell heterogeneity in Bcl-2 expression revealed by ISH staining was confirmed at the protein level. Moderate levels of Bcl-2 protein were detected in most of the cells and stronger immunostaining was found in a small proportion of them. This heterogenous pattern was found in freshly recovered embryos (data not shown) as well as in cultured blastocysts (Fig. 3). Cells with a high immunostaining signal could not be ascribed to a particular cell lineage. In both moderately and strongly positive cells, Bcl-2 protein expression was concentrated in the cytoplasmic compartment. No staining was observed in the blastocoelic cavity. Substitution of the primary antibody with nonimmune rabbit IgG or complete omission of this step from the procedure resulted in very low background staining. Omission of both primary and secondary antibodies indicated that autofluorescence was negligible (Fig. 3).

Total cDNA from blastocysts was amplified with different pairs of Bcl-2 primers. Use of PCR1 and PCR3 generated an amplicon of ~1590 bp in length (data not shown) in accordance with the possibility that a 220-bp putative intron, previously described in the Bcl-2α transcript from other species, is conserved in the rat. Amplification of blastocyst cDNA with PCR2 and PCR3 produced an 887-bp amplicon (Fig. 4) whose sequence was found identical to the corresponding region of the published rat Bcl-2α cDNA. The combination of PCR2 with PCR4, a downstream primer specific for Bcl-2β, generated a 710-bp amplicon whose sequence was identified as corresponding to the second Bcl-2 isoform. Transcripts for both Bcl-2α and Bcl-2β were detected in blastocysts analyzed at the time of collection as well as after culture for 24 h in either 6 mmol/l or 17 mmol/l D-glucose.

Effect of high D-glucose on Bcl-2 expression. Blastocysts incubated in either 6 mmol/l or 28 mmol/l D-glucose for 24 h were compared for their average number of cells per embryo and for the frequencies of cells showing signs of nuclear fragmentation and chromatin degradation. Exposure to high D-glucose resulted in a significant 14% decrease in the mean number of cells per embryo ($P \leq 0.01$) and in a significant threefold increase in the incidence of both nuclear apoptotic markers ($P \leq 0.01$) (Fig. 5). The proportion of cells displaying a mitotic figure remained low (<1%) in both culture groups (data not shown). Incubating the blastocysts in the hyperosmotic control culture medium had no influence compared with 6 mmol/l D-glucose (Fig. 5).

Exposure to high D-glucose for 24 h also induced an eightfold increase in the proportion of cells that were strongly positive for the transcription of the Bcl-2 gene when compared with either 6 mmol/l D-glucose or hyperosmotic control culture medium ($P \leq 0.01$) (Fig. 5). Close inspection of immunostained blastocysts revealed a similar increase in the proportion of cells that were strongly labeled for the Bcl-2 protein after exposure to high D-glucose for 24 h against 6 mmol/l D-glucose (data not shown).

Effect of blocking Bcl-2 synthesis on the blastocyst response to high D-glucose. Antisense oligodeoxynucleotide activity was verified in a cell-free protein synthesis assay using the complete coding sequence of mouse Bcl-2α as a template. In the presence of RNase H, the addition of antisense

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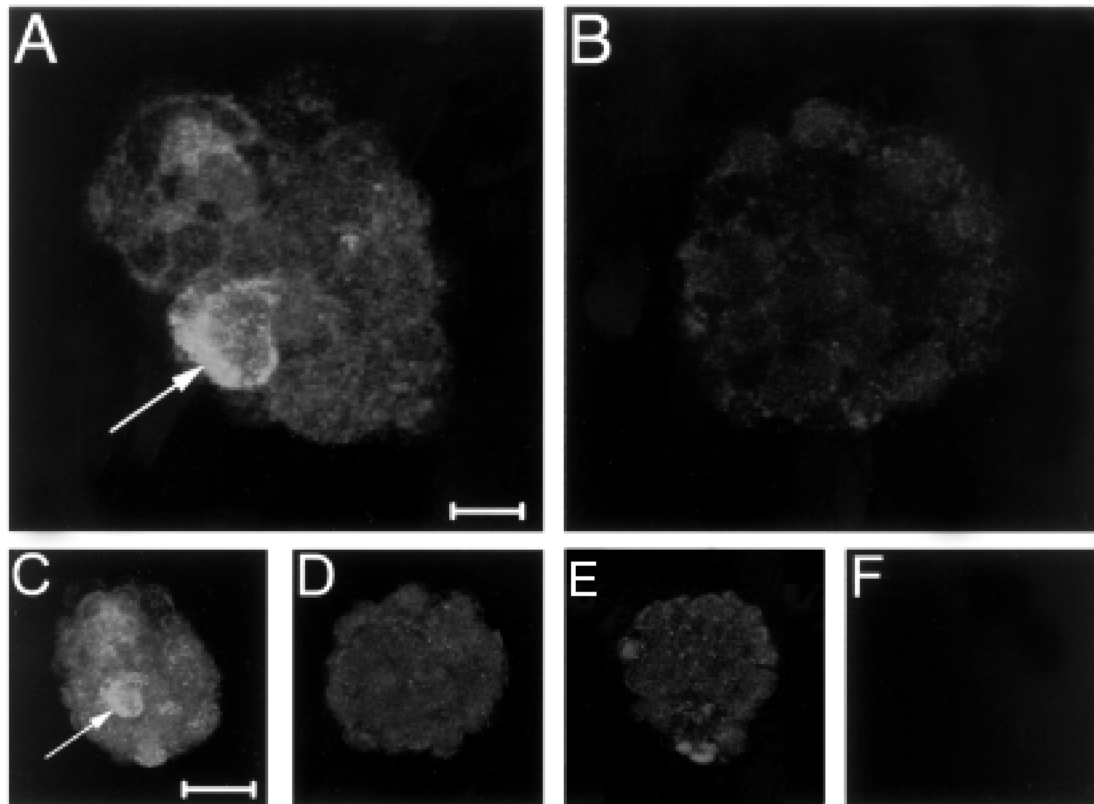

FIG. 3: Bcl-2 protein expression in rat blastocysts. *A*: Immunocytochemistry was performed on a blastocyst after culture for 24 h in 6 mmol/l D-glucose. A primary rabbit antibody against human Bcl-2 and a secondary goat antibody raised against rabbit IgG and coupled to FITC were used. This confocal section shows one strongly Bcl-2⁺ cell (➤) surrounded by less intensively stained cells. Nuclear exclusion of the fluorescent signal is observed in most of the cells. This embryo is representative of a total of 20 blastocysts that were analyzed for Bcl-2 protein expression by immunocytochemistry after culture. *B*: Confocal section of a cultured blastocyst that was processed through the same immunocytochemical procedure except that nonimmune rabbit IgG was used instead of anti–Bcl-2 antibody (negative control). *C*: Z-axis projection of the complete series of sections recorded throughout the blastocyst shown in *A* with the strongly Bcl-2⁺ cell still prominent (➤). *D*: Z-axis projection of the complete series of sections acquired throughout the blastocyst shown in *B*. Additional negative immunocytochemical reactions after the omission of the primary antibody (*E*, Z-axis projection) or omission of both primary and secondary antibodies (*F*, Z-axis projection) show the low fluorescence signals produced by nonspecific antibody binding and autofluorescence, respectively. The scale bar represents 20 µm in *A* and *B* and 45 µm in *C*, *D*, *E*, and *F*.

oligodeoxynucleotides against the BH4 (ASBH4-Ø) or BH2 (ASBH2-Ø) domains of Bcl-2 completely abolished the synthesis of the expected 236 amino acid-long protein, whereas the addition of the corresponding sense oligodeoxynucleotides had no such effect (Fig. 6) (data not shown). The addition of antisense oligodeoxynucleotides against the TM domain of Bcl-2 (ASTM-Ø), in contrast to ASBH4-Ø and ASBH2-Ø, resulted in the reproducible formation of a shorter protein that may correspond to a TM-truncated 209–amino acid version of the protein. The addition of the corresponding sense oligodeoxynucleotide had no effect on the synthesis of the Bcl-2 protein (data not shown). Control reactions with RNase H alone showed that the enzyme had no inhibitory activity on the transcription-translation of the Bcl-2 cDNA template in the absence of antisense oligonucleotides (Fig. 6). In a second series of experiments, rat blastocysts were incubated in the presence or absence of the different antisense oligodeoxynucleotides and then subjected to RT-PCR with the Bcl-2–specific primer pair PCR2 and PCR3. Amplification for Bcl-2 was found to be specifically inhibited by the addition of antisense oligodeoxynucleotides ASBH4-Ø, ASBH2-Ø, or ASTM-Ø, whereas control amplification for either β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could proceed normally (Fig. 6). None of the control sense oligodeoxynucleotides was found to prevent amplification of Bcl-2 mRNA extracted from treated blastocysts (Fig. 6) (data not shown).

The deleterious impact of 28 mmol/l D-glucose on blastocyst development was found to be significantly enhanced when the embryos were cotreated with high D-glucose and antisense oligodeoxynucleotides ASBH4-Ø or ASBH2-Ø. Cell deficiency was 23% after the combination of high D-glucose with either one of these two antisense oligodeoxynucleotides ($P \leq 0.05$ vs. high D-glucose alone) (Fig. 7). This combination also induced a significantly higher increase in the frequency of cells displaying signs of chromatin degradation ($P \leq 0.01$ vs. high D-glucose alone). In contrast, neither ASBH4-Ø nor ASBH2-Ø antisense oligodeoxynucleotides were found to enhance the occurrence of high D-glucose–induced nuclear fragmentation. In control experiments, none of the sense oligodeoxynucleotides had any detectable effect on the different responses of the blastocysts to high D-glucose (Fig. 7).

In contrast to the sensitizing effect of ASBH4-Ø and ASBH2-Ø, antisense oligodeoxynucleotides directed against the TM domain of Bcl-2 improved the resistance of blastocysts to the impact of high D-glucose. Compared with blastocysts incubated in high D-glucose alone, embryos cotreated with high

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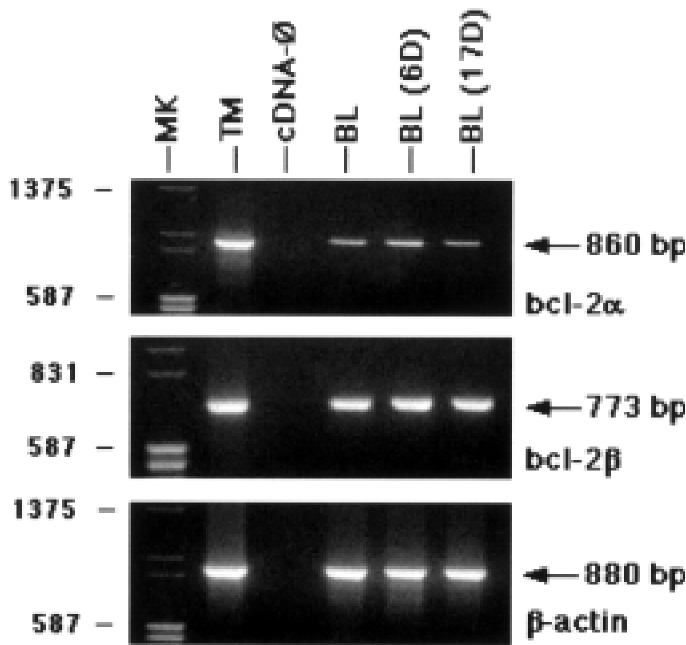

FIG. 4. Bcl-2α and Bcl-2β mRNA in rat blastocysts. Total mRNA was extracted from rat blastocysts, reverse transcribed, and then subjected to amplification using primers designed to selectively amplify the transcripts encoding either Bcl-2α (top panel) or Bcl-2β (middle panel). Bcl-2 mRNA expression was analyzed in blastocysts at the time of collection (BL) and after incubation for 24 h in either 6 mmol/l D-glucose [BL (6D)] or 17 mmol/l D-glucose [BL (17D)]. Positive amplification reactions were run using cDNA from rat thymus (TM), whereas negative reactions were carried out without cDNA input (cDNA-Ø). The cDNA preparations were also analyzed for β-actin (bottom panel). Reaction products were visualized by gel electrophoresis alongside DNA size markers (MK, left margin) and revelation by ethidium bromide staining. Expected amplicon sizes are indicated in the right margin in bps.

D-glucose and ASTM-Ø antisense oligodeoxynucleotides had a significantly lower cell deficit ($P \leq 0.01$) and incidence of chromatin degradation ($P \leq 0.01$) (Fig. 7). However, ASTM-Ø antisense oligodeoxynucleotides did not influence the induction of nuclear fragmentation in blastocysts by high D-glucose.

DISCUSSION

Although Bcl-2 protein synthesis has been detected in preimplantation embryos (9,13) and in derivatives of the three fetal germ layers during early organogenesis (16) in the mouse, Bcl-2–null embryos are able to develop normally throughout the first half-period of gestation (17). However, recent reports on mouse blastocysts indicate that Bcl-2 mRNA may be differentially expressed in the ICM and TE cell lineages (12) and that Bcl-2 protein expression is decreased in fragmented embryos (13), two observations that indirectly support the possibility that Bcl-2 is important during early embryogenesis. In the present study, we show that Bcl-2 is expressed at both mRNA and protein levels in rat blastocysts with no apparent difference between ICM and TE cells. In both cell lineages, a small proportion of cells were found to contain high concentrations of Bcl-2 transcripts and proteins. Heterogeneous distribution of two other Bcl-2–related effectors, Bax and Bcl-X, has previously been described in human blastocysts (18) and interpreted as reflecting the consequence of a cell-specific pattern of gene transcription activity that may be initiated after fertilization (4). A broad range of Bcl-2 expression levels has also been described in human cytotrophoblasts (19), raising the possibility that their sensitivity to cell-death induction may be predetermined based on a Bcl-2–dependent mechanism.

Rat blastocysts reacted to high D-glucose with an increase in the frequencies of two nuclear markers of apoptosis, chromatin degradation (karyolysis) and nuclear fragmentation (karyorhexis), as well as with an increase in the proportion of cells that were strongly labeled for Bcl-2 mRNA and protein

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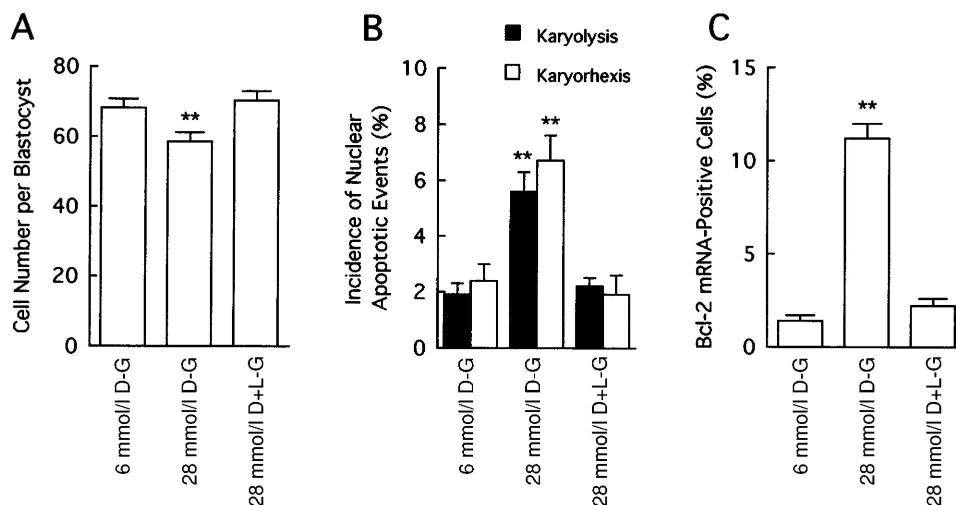

FIG. 5. Effect of high D-glucose on rat blastocysts. *A*: Blastocysts were incubated for 24 h in either 6 mmol/l D-glucose (6 mmol/l D-G), 28 mmol/l D-glucose (28 mmol/l D-G), or a combination of 6 mmol/l D-glucose and 22 mmol/l L-glucose (28 mmol/l D+L-G) and then counted for their average number of cells per embryo. Between 30 and 40 embryos were analyzed per culture condition. *B*: The same blastocysts as in *A* were also examined for the percentages of cells featuring either one of two nuclear apoptotic events, chromatin degradation (■, karyolysis) or nuclear fragmentation (□, karyorhexis). The dual nuclear staining technique used to simultaneously reveal the presence of nuclei engaged in either karyolysis or karyorhexis in these embryos is described in the text. *C*: Blastocysts were incubated as in *A* and analyzed by in situ hybridization for the percentage of cells strongly positive for Bcl-2 mRNA expression. Between 30 and 35 embryos were examined per culture condition. **Statistically significant difference from control values in 6 mmol/l D-G at $P \leq 0.01$.

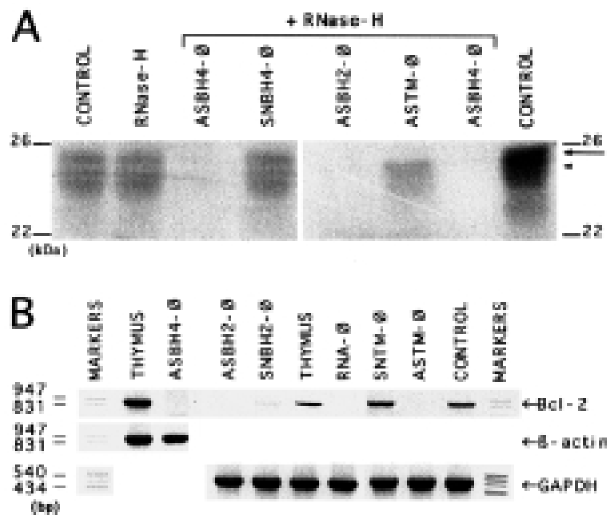

FIG. 6. Influence of antisense oligodeoxynucleotides on Bcl-2 expression. *A*: An acellular transcription-translation assay was performed using a cDNA encoding the complete sequence of mouse Bcl-2α as template. Reactions were performed under control conditions (CONTROL), in the presence of ribonuclease-H alone (RNase-H) or in the presence of ribonuclease-H and either one of three different antisense oligodeoxynucleotides directed against the corresponding BH4, BH2, and TM domains of the Bcl-2 protein (ASBH4-Ø, ASBH2-Ø, and ASTM-Ø, respectively). In this experiment, a control negative reaction was carried out in parallel using sense oligodeoxynucleotide SNBH4-Ø combined with ribonuclease-H. Control negative reactions with the sense oligodeoxynucleotides SNBH2-Ø and SNTM-Ø were performed in other series of experiments (data not shown). Reaction products were visualized by Western blot analysis using a rabbit primary anti-mouse Bcl-2 antibody alongside protein size markers (left margin, kDa). Complete Bcl-2 protein size is 236 a.a. (right margin, ➤) and truncated Bcl-2 protein size is 209 a.a. (➤). *B*: Total cDNA was prepared from rat thymus (THYMUS) and from rat blastocysts after incubation for 24 h in either control culture medium (CONTROL) or in the presence of either one of the three antisense oligodeoxynucleotides ASBH4-Ø, ASBH2-Ø, and ASTM-Ø. Control negative tests were carried out in parallel with sense oligodeoxynucleotides SNBH2-Ø and SNTM-Ø. The absence of influence of sense oligodeoxynucleotide SNBH4-Ø was verified in other series of experiments (data not shown). The cDNAs were amplified with primers specific for Bcl-2α, β-actin, or GAPDH (right margin) and the reaction products were analyzed by gel electrophoresis alongside DNA size markers (left margin, in bp). A control negative amplification was performed in parallel without RNA input during the reverse-transcription step (RNA-Ø). In the course of the experiments summarized above, each antisense or sense oligodeoxynucleotide was tested at least twice with identical results (effect or lack thereof) in the acellular assay as well as in blastocysts.

expression. Transient increases in mRNA and protein Bcl-2 expression levels have been observed in reaction to the induction of apoptosis in several cell systems, such as in pancreatic β-cell lines on serum withdrawal (20), supporting the concept that elevation in the production of antiapoptotic effectors may be integral to the survival of certain cells. Interestingly, mouse blastocysts have previously been found to react to an exposure to high D-glucose with an increase in pro-apoptotic Bax expression (11). However, in contrast to Bcl-2, enhanced Bax expression was evenly distributed across the embryos, suggesting that antagonizing Bcl-2 and Bax effectors may be differentially up-regulated by high D-glucose in embryonic cells.

Previous studies have shown that Bcl-2 can occur in two alternatively spliced isoforms that differ at their C-termini (21). Compared with shorter Bcl-2β, Bcl-2α contains a BH2 domain that is crucial for dimerization with other Bcl-2–like proteins and a transmembrane domain that anchors Bcl-2α to intracellular membranes. In the present study, RT-PCR analysis revealed that both Bcl-2α and Bcl-2β isoforms were coexpressed in rat blastocysts. Preliminary data were obtained that suggested a preferential upregulation of Bcl-2α mRNA in blastocysts exposed to high D-glucose (data not shown), but more experiments will be required to confirm that observation.

To better delineate the relevance of Bcl-2 in the embryonic response to high D-glucose, rat blastocysts were pretreated with antisense oligodeoxynucleotides directed against different sequences of the Bcl-2 mRNA, which correspond to distinct functional domains in the Bcl-2α protein. The first

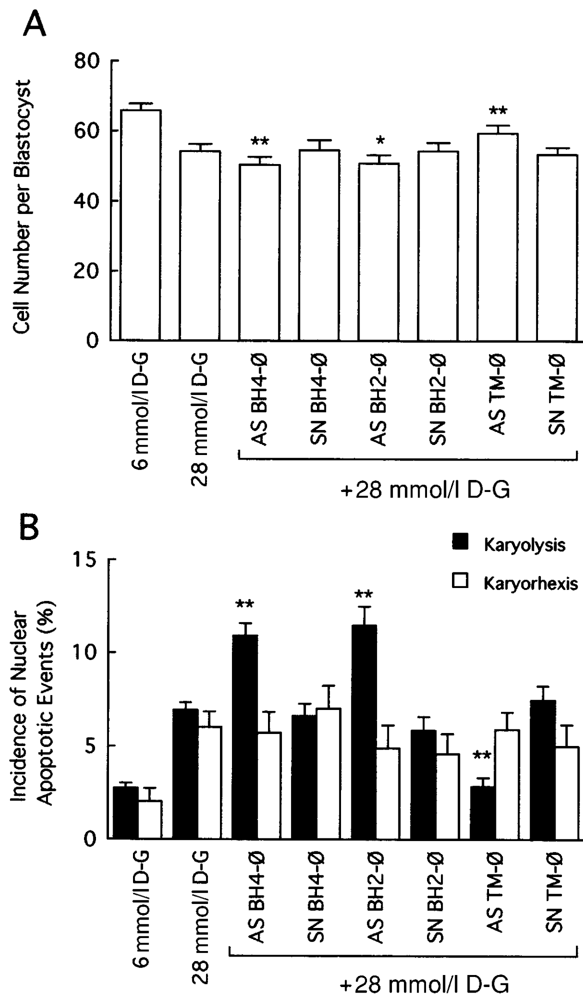

FIG. 7. Effect of antisense oligodeoxynucleotides on cell proliferation and apoptosis in rat blastocysts. *A*: Blastocysts were incubated for 24 h in either 6 mmol/l D-glucose (6 mmol/l D-G), 28 mmol/l D-glucose (28 mmol/l D-G) or 28 mmol/l D-glucose combined with either one of the antisense or sense oligodeoxynucleotides pretested in Fig. 6. At the end of the incubation period, the blastocysts were counted for their average number of cells per embryo. Between 25 and 40 embryos were analyzed per culture condition. *B*: The same blastocysts as in *A* were also examined for the percentages of cells featuring either one of two nuclear apoptotic events, chromatin degradation (■, karyolysis) and nuclear fragmentation (□, karyorhexis). The dual nuclear-staining technique used to simultaneously reveal the presence of nuclei in either karyolysis or karyorhexis in these embryos is described in the text. $^*P \leq 0.05$, $^{**}P \leq 0.01$, statistically significant difference from average values in 28 mmol/l D-G alone.

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antisense oligodeoxynucleotide, ASBH4-Ø, was designed to hybridize to the BH4 domain that is common to the two Bcl-2 isoforms. Pretreatment with ASBH4-Ø was found to decrease the expression of Bcl-2 and to sensitize the blastocysts to the impact of high D-glucose on cell proliferation and chromatin degradation. The addition of ASBH2-Ø, an oligodeoxynucleotide against the BH2 dimerization domain that is absent in Bcl-2β, had the same effects as ASBH4-Ø. Thus, in support of the general hypothesis that Bcl-2 is an antiapoptotic protein, inhibiting its synthesis resulted in increased D-glucose embryotoxicity.

In contrast, the addition of ASTM-Ø, an oligodeoxynucleotide hybridizing just upstream of the Bcl-2α transmembrane domain, protected the blastocysts against high D-glucose. In vitro protein synthesis assays showed that production of a smaller Bcl-2 protein occurred in the presence of ASTM-Ø, suggesting that hybridization of ASTM-Ø to Bcl-2 transcripts would not lead to mRNA degradation but rather induce modifications in Bcl-2 mRNA splicing (22) or block the completion of later translational events (23) and thereby produce a truncated Bcl-2 protein. The former possibility may also explain the absence of Bcl-2 amplicon when mRNA from ASTM-Ø–treated blastocysts was examined by RT-PCR. Previous studies have shown that Bcl-2α proteins lacking a transmembrane domain can increase cell resistance to cytotoxic agents under certain conditions (24,25). Whether this explanation applies in ASTM-Ø–treated blastocysts remains to be investigated.

None of the anti–Bcl-2 antisense oligodeoxynucleotides influenced the susceptibility of rat blastocysts to the induction of nuclear fragmentation by high D-glucose. This suggests that, in contrast to chromatin degradation, Bcl-2 is not involved in this second nuclear apoptotic event. Experiments in progress show that specific inhibition of either caspase-3 or caspase-activated deoxyribonuclease in rat blastocysts also failed to block the induction of nuclear fragmentation by high D-glucose (data not shown), an indication that the intracellular cascades leading to nuclear fragmentation and chromatin degradation may be either completely independent or diverge downstream of a common trigger mechanism.

There is now convincing evidence that severe developmental anomalies leading to fetal resorption or malformation can occur as consequences of subtle damages inflicted to the embryos before or at the time of implantation (26). One of these primary damages may be the disruption of the highly regulated gene program that controls the expression pattern of crucial developmental determinants during early embryogenesis, including apoptosis (9). The present study shows that rat blastocysts express the antiapoptotic effector Bcl-2 and that their exposure to high D-glucose in vitro induces an increase in Bcl-2 expression in a limited number of cells. Blocking Bcl-2 synthesis with antisense oligodeoxynucleotides sensitized the blastocysts to the apoptotic impact of D-glucose. If the implication of Bax (11) and Bcl-2 in the control of the apoptotic cascade that is triggered by high D-glucose in blastocysts thus appears established, then the precise nature of the signals that are acting upstream and downstream of these effectors remains to be investigated.

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