Placenta 90 (2020) 27–36

Contents lists available at ScienceDirect

Placenta

journal homepage: http://www.elsevier.com/locate/placenta

# Decreased placental apoptosis and autophagy in pregnancies complicated by gestational diabetes with large-for-gestational age fetuses

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ARTICLE INFO

*Keywords:*
Apoptosis
Autophagy
Bcl-2 family proteins
Gestational diabetes mellitus
Placenta

ABSTRACT

*Introduction:* Dysregulation of placental apoptosis and autophagy are observed in pregnancy complications including preeclampsia and fetal growth restriction. However, studies of their changes in the placentas of women with gestational diabetes mellitus (GDM) show inconsistent results. We aimed to compare the changes in apoptosis, autophagy, and Bcl-2 family proteins in the placentas from women with normal pregnancies and those with GDM, with or without large-for-gestational age (LGA) infants and to investigate the effect of hyperglycemia on the changes in apoptosis, autophagy, and Bcl-2 family proteins in primary cytotrophoblastic cells.
*Methods:* Villous tissues were obtained from normal pregnant women and those with GDM, with or without LGA infants. Primary cytotrophoblast cells were isolated from normal term placentas and cultured under standard, hyperglycemic, or hyperosmotic conditions.
*Results:* Compared to placentas from normal pregnant women, those from GDM women with LGA infants were heavier, had lower beclin-1 and DRAM levels, less M30 and cleaved PARP immunoreactivity, and increased Ki-67 immunoreactivity. These changes were associated with increased Bcl-xL and decreased Bak levels. Increased glucose concentration led to lower ATG5, beclin-1, LC3B-II, p62, and DRAM levels, lower annexin V and M30-positive cell percentages, and less cleaved PARP changes compared with standard culture conditions. Hyperglycemia caused higher Bcl-xL levels and lower Bak and Bad levels than did standard culture conditions.
*Discussion:* There were differential changes in apoptosis and autophagy between placentas from normal pregnant women and those from GDM women with LGA infants. Bcl-2 family proteins are likely involved in the regulation of these changes.

## 1. Introduction

Women with gestational diabetes mellitus (GDM) are at increased risk of obstetrical complications including preeclampsia, shoulder dystocia, and birth injury and are more likely to require operative vaginal and cesarean deliveries for large-for-gestational age (LGA) infants [1]. Neonates of GDM pregnancies are also at risk of hypoglycemia, hyperbilirubinemia, and respiratory distress syndrome. The hyperglycemic intrauterine environment in GDM women affects fetal and placental development, and placental function [2–4].

The human placenta is a highly specialized organ in the interface between maternal and fetal circulation with fundamental functions for pregnancy. Among placental cells, trophoblasts permit embryo implantation and nutrition in early pregnancy following which they contribute considerably to the development and function of the placenta. Compared to those from women with normal pregnancies, placentas from GDM women are usually bigger and heavier, suggestive of alterations in trophoblast cell proliferation, differentiation, and death [5]. However, the exact mechanisms accounting for the increased placental mass remain unclear. Some evidence shows increased immunoreactivity of cellular proliferation markers including proliferative cell nuclear antigen (PCNA) and Ki-67 in the villous cytotrophoblasts, syncytiotrophoblasts, stromal cells, and endothelial cells in diabetic placentas [6]. Furthermore, a higher number of villous cytotrophoblasts

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https://doi.org/10.1016/j.placenta.2019.12.003
Received 26 May 2019; Received in revised form 21 November 2019; Accepted 3 December 2019
Available online 3 December 2019

and syncytiotrophoblast nuclei are observed in diabetic placentas than in normal placentas [5,7]. These changes may contribute to increased placental size.

Alternatively, increasing placental mass in GDM pregnancy may be caused by dysregulation of trophoblast cell death. Apoptosis (programmed cell death) and autophagy (a catabolic process involving invagination and degradation of cytoplasmic components through the lysosomal pathway) are well-controlled biological processes that play essential roles in development, tissue homeostasis, and disease. Autophagy and apoptosis are often co-activated in response to stress and both have been implicated in various physiological or pathological processes including cellular differentiation and cell death. Both apoptosis and autophagy participate in the development of the human placenta and increased apoptosis and autophagy have been observed in pregnancy complications such as preeclampsia and fetal growth restriction [8–13]. However, the roles of apoptosis and autophagy in GDM placentas are not clear. Some studies show a decrease in apoptosis in GDM placentas compared to that in placentas of normal pregnancies [14,15], while others do not [16–18]. Only a few studies, with inconsistent results, have been conducted that investigate autophagic changes in GDM placentas [19,20].

Compared with GDM women with appropriate-for-gestational age (AGA) infants, GDM women with LGA infants have a higher rate of prepregnancy obesity, previous macrosomia, combined changes in oral glucose tolerance tests (OGTT), and suboptimal blood glucose control in the third trimester [21]. GDM women with LGA infants are also at a higher risk of adverse pregnancy outcomes, including cesarean delivery for prolonged labor, shoulder dystocia, neonatal hypoglycemia, and jaundice, than GDM women with AGA infants [22]. These differences in maternal characteristics and pregnancy outcomes have led us to hypothesize that there may be differential changes in apoptosis and autophagy in placentas between GDM women with and without LGA infants.

Therefore, this study was designed to investigate changes in apoptosis and autophagy in placentas between normal pregnancies and pregnancies complicated by GDM with or without LGA fetuses. Specifically, we aimed to compare the placental apoptotic and autophagic changes between women with normal pregnancies and those with pregnancies complicated by GDM, with or without LGA infants, and to study the apoptotic and autophagic changes between cells cultured under standard and hyperglycemic conditions. Furthermore, increasing evidence shows that Bcl-2 family proteins play essential roles in the regulation of autophagy and apoptosis [23]. We compared changes in Bcl-2 family protein levels, including Bcl-2, Bcl-xL, Bax, Bak, and Bad, in the placentas of normal pregnant women and those with GDM and LGA infants. We then investigated the roles of Bcl-2 family proteins in the regulation of apoptosis and autophagy in trophoblastic cells under hyperglycemic conditions.

## 2. Materials and methods

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Taiwan (IRB No. 201509551B0 and 201702210B0). All placental samples were collected after the subjects enrolled herein provided written informed consent for the use of the samples. Unless otherwise indicated, the reagents used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.1. Collection of placental tissues from normal pregnancies and pregnancies complicated by GDM

We obtained placentas from women with singleton term pregnancies who underwent elective cesarean deliveries prior to the onset of labor to compare the levels of apoptosis and autophagy-related molecules between women with normal pregnancies and AGA infants (n = 10) and women with pregnancies complicated by GDM with AGA (n = 10) or LGA infants (n = 10). Indications for cesarean deliveries included prior cesarean section or myomectomy, cephalopelvic disproportion, and fetal malpresentation. AGA is defined as a birth weight between the 10th and 90th percentiles while LGA is defined as a birth weight above the 90th percentile for the mean weight corrected for fetal sex and gestational age, respectively. In Taipei Chang Gung Memorial Hospital, universal screening for GDM is usually performed between 24 and 28 weeks of gestation with the 75-g, 2-h, OGTT. GDM is diagnosed when one or more of the following plasma glucose levels is met or exceeded: fasting, 92 mg/dL; 1-h, 180 mg/dL; or 2-h, 153 mg/dL [24]. None of these 30 women had any other medical diseases, such as preeclampsia, renal, or autoimmune diseases.

We randomly collected villous tissue samples from five distinct sites on the maternal side of the placenta after it was delivered. Each site was midway between the cord insertion site and placenta periphery and midway between the chorionic and basal plates. The villous samples were quickly rinsed in ice-cold phosphate-buffered saline (PBS) to clear the maternal blood and were then either fixed in 4% paraformaldehyde or snap frozen in liquid nitrogen before being stored at −70 °C for further processing. All villous samples were collected and processed within 10 min after delivery.

### 2.2. Isolation and culture of cytotrophoblast cells from normal term placentas

We isolated cytotrophoblast cells from 10 normal term placentas as previously described [25]. The purified cells were plated in 6-well plates at a minimum density of $4 \times 10^6$ cells/cm$^2$ and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco; catalog no. 12320032; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5.6 mM D-glucose, 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (antibiotic-antimycotic, catalog no. 15240062; Gibco) in a humidified atmosphere with 5% $CO_2$ and balanced air. After an overnight rest, the cells were rinsed twice with pre-warmed medium to remove non-attached cells and then used in individual experiments. The quality and purity of the cytotrophoblasts were routinely checked by immunofluorescence of cytokeratin-7 and measurement of beta-hCG in the medium as previously reported [25].

To study the effect of increasing glucose concentrations on the changes of apoptosis, autophagy, and proliferation, cytotrophoblasts were cultured in a medium with one of the following three glucose concentrations: (1) 5.6 mM D-glucose (defined as the standard condition), (2) 5.6 mM D-glucose and 19.4 mM L-glucose (defined as the hyperosmotic control), and (3) 25 mM D-glucose (defined as the hyperglycemic condition; DMEM; catalog no. 12430062; Gibco) in a humidified chamber with 5% $CO_2$/balanced air, respectively. After 24 h of incubation, cells were collected for flow cytometry or were homogenized and stored at −70 °C for further processing.

### 2.3. Western blot analysis

Western blotting was performed as previously described [11]. Briefly, villous tissues and cytotrophoblastic cells were homogenized and lysed in ice-cold protein extraction reagents (T-PER reagent and M-PER reagent; Pierce Biotechnology, Rockford, IL, USA) with complete miniprotease inhibitor cocktail (Roche). Lysates were centrifuged at 10,000×*g* at 4 °C for 20 min, after which the supernatants were decanted off. Protein concentrations were determined based on the Bradford dye-binding procedure using a protein assay kit (catalog no. 500–0002; Bio-Rad Laboratories, Hercules, CA, USA). Fifty to one hundred micrograms of cytosolic protein sample per lane were separated by 12% or 16% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with primary antibodies overnight at 4 °C. The relative intensities of the protein signals were normalized to the intensities of the β-actin signals, and the band densities were quantified by densitometric analysis using ImageJ software

(National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). The source and working concentration of each primary antibody are listed in Table 1.

### 2.4. Real-time quantitative PCR

Real-time quantitative PCR analysis was performed as previously described [26]. Assay-on-Demand TaqMan primers and probes for human *ATG5* (Hs00169468_m1), *BECN1* (Hs00186838_m1), *LC3B* (Hs00797944_s1), *p62* (Hs02621446_s1), *DRAM1* (Hs00218048_m1), *Bcl-2* (Hs00608023_m1), *Bcl-xL* (Hs00236329_m1), *Bax* (Hs00751844_s1), *Bak1* (Hs00832876_g1), and *Bad* (Hs00188930_m1) from Applied Biosystems (Life Technologies, Grand Island, NY, USA) were used. 18S ribosomal RNA (Hs99999901_s1) was used as an endogenous control because it has been found to remain stable in normoglycemic and hyperglycemic conditions in several types of cells [27,28] and in our preliminary experiments (Supplementary Tables 1-3 and Fig. S1). Thermal cycling was initiated with a 2-min incubation at 50 °C, followed by a first denaturation step of 10 min at 95 °C, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were analyzed on the same run, and each sample was run in triplicate. Relative quantities of target genes and 18S ribosomal RNA were calculated by the comparative threshold cycle (Ct) method as previously described [9].

### 2.5. Immunohistochemistry for M30 (cytokeratin 18 neoepitope), cleaved poly (ADP-ribose) polymerase (PARP), and Ki-67

Immunohistochemistry for M30, cleaved PARP, and Ki-67 was performed as previously described [6]. Briefly, paraformaldehyde-fixed, paraffin-embedded sections were dewaxed in xylene twice for 5 min each and then serially rehydrated for 5 min each in decreasing concentrations of ethanol (100%, 90%, 70%, and 50%, followed by distilled water) before immunohistochemical staining. Sections were immersed in 10 mM sodium citrate buffer (pH 6.0) and autoclaved (120 °C for 20 min) for antigen retrieval. After cooling to room temperature, endogenous peroxidase activity was quenched with 3% $H_2O_2$ in methanol for 10 min, nonspecific binding was blocked with 10% normal serum from the same species as the secondary antibody in PBS with 0.1% Tween-20 for 30 min, and sections were reacted with the primary antibodies listed in Table 1 at 4 °C overnight. Further processing for colorimetric detection was according to the instructions for the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine as the peroxidase substrate. Counterstaining with hematoxylin was carried out if needed. Negative controls included substitution of the primary antibody with non-immune rabbit IgG or mouse isotypic IgG. With a 40 × objective, sections were viewed and photographed. Five randomly selected fields for each section were examined by three investigators who were blinded to the identity of the tissue. The section was scored for intensity (absent, faint, moderate, or intense [0 through 3]) of M30 immunostaining, and counted for the percentage of cleaved PARP or Ki-67-positively stained cells in the trophoblast layer.

### 2.6. Flow cytometry

Following individual experiments, cells were collected and single-cell suspensions were prepared for intracellular staining with M30 and Ki-67 after treatment with fixation and permeabilization buffers (catalog no. 00–8222 and 00–8333, eBioscience, Level Biotechnology, New Taipei City, Taiwan) according to the manufacturer's protocols. Acquisition was performed using FACSCalibur (BD Biosciences, Taipei, Taiwan), and data analysis was conducted using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

### 2.7. Statistical analysis

The data of the characteristics of the study population were presented as the mean ± SD, median (range), or number (%) and data of the levels of apoptosis and autophagy-related molecules were presented as mean ± SEM. They were analyzed and plotted using Prism 7 for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA). Differences between groups were determined with the one-way analysis of variance followed by Bonferroni's test or the Kruskal-Wallis test followed by Dunn's multiple comparison test. Results with $P$ values of less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Clinical characteristics of the study population

The characteristics of the women who participated in this study and their pregnancies are summarized in Table 2. Three of the 10 GDM women with AGA infants and two of the 10 GDM women with LGA infants underwent insulin treatment because of unsatisfactory blood glucose control with nutritional therapy. All women with GDM had normal 75 g OGTTs 6–8 weeks postpartum. Compared to women with normal pregnancies, those with GDM and LGA infants had significantly higher 75 g OGTT 1 and 2-h glucose levels, birth weights, and placental weights. Furthermore, GDM women with LGA infants had a lower birth weight to placental weight ratio with marginal significance compared with the other two groups of women ($P = 0.05$). Women with GDM and AGA infants also had a significantly higher 2-h OGTT glucose levels than did those without GDM. Moreover, GDM women tended to have higher systolic blood pressure (at the first trimester and at delivery) and pre-pregnancy body mass indexes than normal pregnant women, although the differences were not significant. Otherwise, no differences in mean gestational age at delivery and other characteristics, including maternal

**Table 1**
Primary antibodies used in the western blot and immunohistochemistry.

| Antibody | Manufacturer | Source | Type | Catalog no. | Dilution |
|---|---|---|---|---|---|
| ATG5/APG5L | Abcam | Rabbit | Polyclonal | Ab108327 | WB 1:5000 |
| Beclin-1 | Cell signaling | Rabbit | Polyclonal | 3738 | WB 1:500 |
| LC3B | Cell signaling | Rabbit | Polyclonal | 2775 | WB 1:200 |
| p62/SQSTM1 | Cell signaling | Rabbit | Polyclonal | 5114 | WB 1:500 |
| DRAM (M3-P4B4) | Santa cruz | Mouse | Monoclonal $IgG_1$ | SC-81713 | WB 1:1000 |
| Bcl-2 (N-19) | Santa cruz | Rabbit | Polyclonal | SC-492 | WB 1:200 |
| Bcl-xL (H-5) | Santa cruz | Mouse | Monoclonal $IgG_1$ | SC-8392 | WB 1:100 |
| Bax | Cell signaling | Rabbit | Polyclonal | 2772 | WB 1:1000 |
| Bak (G-23) | Santa cruz | Rabbit | Polyclonal | SC-832 | WB 1:500 |
| Bad (C-7) | Santa cruz | Mouse | Monoclonal $IgG_1$ | SC-8044 | WB 1:100 |
| β-actin (AC-74) | Sigma | Mouse | Monoclonal $IgG_{2a}$ | A5316 | WB 1:10000 |
| M30 CytoDEATH | Roche | Mouse | Monoclonal $IgG_{2b}$ | 12140322001 | IHC 1:100 |
| Cleaved PARP (Asp214) | Cell signaling | Rabbit | Monoclonal IgG | 5625 | IHC 1:50 |
| Ki-67 | Abcam | Rabbit | Polyclonal | Ab15580 | IHC 1:2000 |

WB, western blot; IHC, immunohistochemistry.

**Table 2**
Characteristics of the study population.

| | Normal pregnancy (n = 10) | GDM with AGA (n = 10) | GDM with LGA (n = 10) | *P* |
|---|---|---|---|---|
| Age (y) | 36.6 ± 3.6 | 37.4 ± 2.8 | 35.5 ± 3.3 | 0.46 |
| Primiparity | 5 (50%) | 5 (50%) | 7 (70%) | 0.58 |
| Prepregnancy body mass index (kg/m$^2$) | 20.7 ± 1.9 | 23.0 ± 2.8 | 24.0 ± 4.3 | 0.07 |
| Blood pressure at first trimester | | | | |
| Systolic (mm Hg) | 105 ± 10 | 119 ± 10 | 116 ± 16 | 0.05 |
| Diastolic (mm Hg) | 59 ± 6 | 66 ± 9 | 66 ± 13 | 0.24 |
| Blood pressure at delivery | | | | |
| Systolic (mm Hg) | 120 ± 10 | 131 ± 8 | 123 ± 12 | 0.06 |
| Diastolic (mm Hg) | 76 ± 8 | 81 ± 6 | 78 ± 9 | 0.34 |
| Hemoglobin (mg/dl) | 11.1 ± 1.4 | 11.9 ± 1.7 | 11.5 ± 1.4 | 0.44 |
| Platelet count ($10^3$/μl) | 231 ± 58 | 207 ± 34 | 230 ± 57 | 0.52 |
| Glucose level at 75-g OGTT at 24–28 weeks of gestation (mg/dl) | | | | |
| Fasting | 82 ± 6 | 85 ± 8 | 90 ± 7 | 0.09 |
| 1-hour | 146 ± 11 | 167 ± 28 | 181 ± 20[a] | <0.01 |
| 2-hour | 121 ± 13 | 158 ± 18[b] | 193±9[b] | <0.001 |
| Glucose level at 75-g OGTT 6–8 weeks postpartum (mg/dl) | | | | |
| Fasting | NA | 91 ± 9 | 93 ± 9 | 0.42 |
| 2-hour | NA | 127 ± 27 | 113 ± 22 | 0.13 |
| Insulin therapy | 0 | 3 (30%) | 2 (20%) | 0.19 |
| Gestational age (wk) | 38.1 ± 0.6 | 38.9 ± 1.1 | 39.0 ± 1.2 | 0.13 |
| Birth weight (g) | 3024 ± 106 | 3148 ± 245 | 3892 ± 283[b,c] | <0.001 |
| Placental weight (g) | 599 ± 75 | 594 ± 105 | 833 ± 79[b,c] | <0.001 |
| Birth weight to placental weight ratio | 5.3 ± 0.5 | 5.3 ± 0.8 | 4.7 ± 0.4 | 0.05 |
| One-minute Apgar score | 9 (9–10) | 9 (8–9) | 9 (8–9) | 0.19 |
| Five-minute Apgar score | 10 | 10 (9–10) | 10 (9–10) | 0.37 |
| Male fetus | 5 (50%) | 6 (60%) | 6 (60%) | 0.87 |

OGTT, oral glucose tolerance test; NA, not available; GDM, gestational diabetes mellitus; AGA, appropriate-for-gestational age infants; LGA, large-for-gestational age infants.
Data presented as the mean ± SD, median (range), or n (%).
[a] $P < 0.01$.
[b] $P < 0.001$, compared to women with a normal pregnancy.
[c] $P < 0.001$ compared to GDM women with AGA infants.

age, percentage of primiparity, diastolic blood pressure at the first trimester or delivery, hemoglobin level, and platelet count before delivery, were observed among the three groups.

### 3.2. Differences in the levels of autophagy-related molecules between placentas from normal pregnancies and those from pregnancies complicated by GDM with AGA or LGA infants

ATG5, beclin-1, LC3B-II, DRAM, and p62 were used as autophagy markers. These factors were selected as they represent different functional aspects of the autophagic change [29]. The levels of beclin-1 and DRAM were significantly lower in villous samples from women with pregnancies complicated by GDM with LGA infants compared to the samples from women with normal pregnancies or from women with GDM and AGA infants (Fig. 1b and d). In contrast, the p62 level was significantly higher in placentas from women with GDM and LGA infants than in those from normal pregnancies (Fig. 1e). No differences in the levels of ATG5, beclin-1, LC3B-II, DRAM, and p62 were observed between placentas from women with normal pregnancies and in those from women with GDM and AGA infants.

*ATG5*, *BECN1*, *LC3B*, *DRAM1*, and *p62* mRNA expression was assessed in the three groups of women (Fig. 1f–j). Villous samples from women with pregnancies complicated by GDM with LGA infants displayed significantly lower *BECN1*, *LC3B*, *DRAM1*, and *p62* mRNA levels than did the samples from women with normal pregnancies (Fig. 1g–j). GDM women with LGA infants also had lower levels of *BECN1*, *LC3B*, and *p62* mRNA than did GDM women with AGA infants (Fig. 1g, h, and 1j). There were no differences in the levels of *ATG5*, *BECN1*, *LC3B*, *DRAM1*, and *p62* mRNA between women with normal pregnancies and women with GDM and AGA infants.

### 3.3. Differences in the changes of trophoblastic apoptosis and proliferation between placentas from normal pregnancies and those from pregnancies complicated by GDM with AGA or LGA infants

Immunohistochemistry for markers of apoptosis (M30 and cleaved PARP) and proliferation (Ki-67) was performed (Fig. 2). PARP plays an important role in DNA repair processes, and cleavage of PARP and cytokeratin 18 are downstream events from caspase proteolytic action. M30 immunostaining was observed in some nuclei in the trophoblast layer (Fig. 2a–c, arrows). Compared with normal pregnant women and GDM women with AGA infants, the number of anti-M30 antibody-stained nuclei was lower in GDM women with LGA infants (Fig. 2a–c). Immunoreactivity of cleaved PARP was also noted in focal areas of syncytiotrophoblast and some cytotrophoblasts. Similarly, the immunostaining of cleaved PARP was less extensive, and the number of anti-cleaved PARP antibody-stained nuclei was lower in GDM women with LGA infants than in women with normal pregnancies and in GDM women with AGA infants (Fig. 2e–g). In contrast, villous samples from GDM women with LGA infants showed more cells, including cytotrophoblasts, endothelial cells, and stromal cells, that were positively stained for anti-Ki-67 antibody than villous samples from the other two groups of women (Fig. 2i–k). There was essentially no staining on the negative controls when the primary antibody was substituted with non-immune IgG (Fig. 2d, h, and 2l). Semi-quantitative analysis confirmed the immunohistochemistry results (Table 3).

### 3.4. Differences in Bcl-2 family protein levels between placentas from normal pregnancies and those from pregnancies complicated by GDM with AGA or LGA infants

Bcl-2 family proteins have been implicated in the regulation of autophagy and apoptosis [23]. We studied the changes in the placental levels of anti-apoptotic (Bcl-2 and Bcl-xL) and pro-apoptotic (Bax, Bak, and Bad) molecules in women with normal pregnancies and those with pregnancies complicated by GDM with AGA or LGA infants. Compared to placentas from normal pregnant women, those from GDM women with LGA infants had a significantly higher level of Bcl-xL (Fig. 3b). However, the levels of Bcl-2 and Bcl-xL did not differ between placentas from normal pregnant women and those from GDM women with AGA infants (Fig. 3a and b). In contrast, placentas from GDM women with LGA infants had significantly lower Bak levels than did those from normal pregnant women and GDM women with AGA infants (Fig. 3d). However, there was no difference in the levels of Bax and Bad between the three groups of women (Fig. 3c and e).

We were not able to detect any significant difference in the expression of *Bcl-2* and *Bcl-xL* mRNA between normal pregnant women and women with pregnancies complicated by GDM with either AGA or LGA infants. The observed *Bax*, *Bak*, and *Bad* mRNA expression essentially reflects the changes in protein levels. GDM women with LGA infants had significantly lower *Bak* mRNA levels than did normal pregnant women and GDM women with AGA infants (Fig. 3i) and no difference was observed in *Bax* and *Bad* mRNA levels between the three groups (Fig. 3h and j).

### 3.5. High glucose concentration is associated with decreased autophagic changes in cytotrophoblast cells

To investigate the effect of increasing glucose concentration on autophagic changes, primary cytotrophoblast cells were cultured in

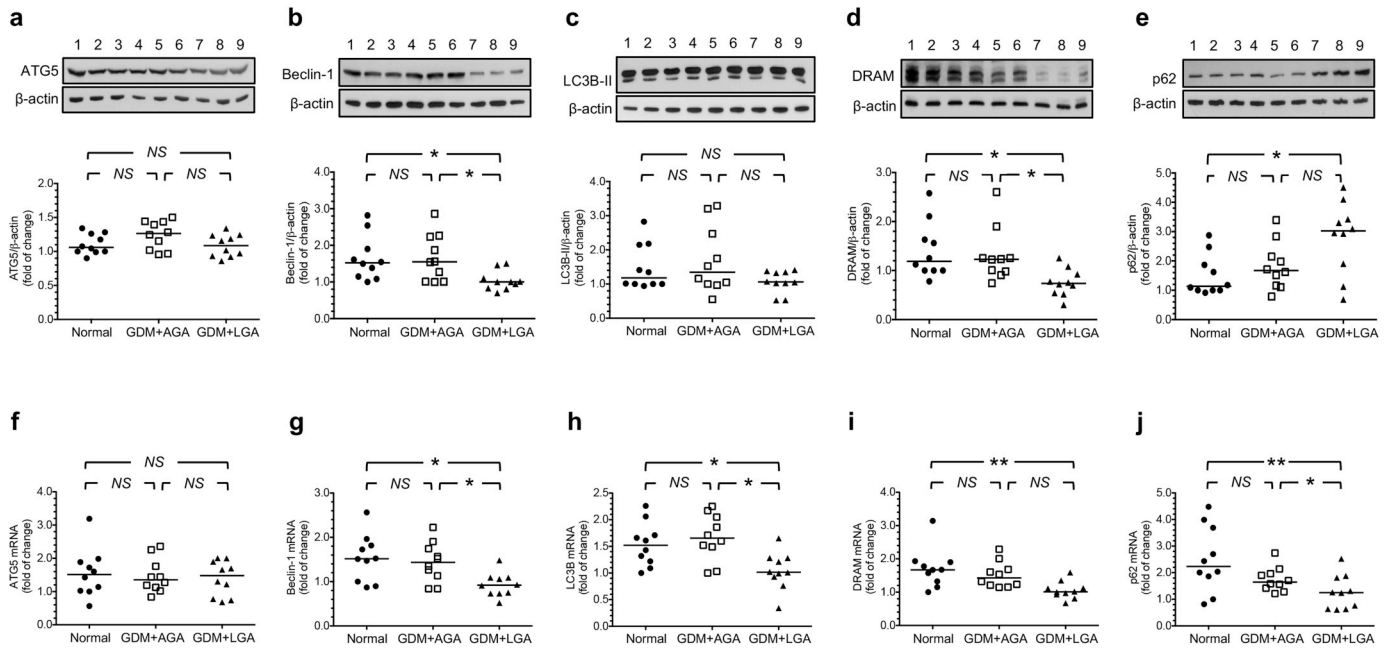


**Fig. 1. Differences in the translational and transcriptional levels of autophagy-related molecules including ATG5, beclin-1, LC3B-II, DRAM, and p62 between placentas from normal pregnancies and those from pregnancies complicated by GDM with AGA or LGA infants.** Levels of beclin-1 (b) and DRAM (d) were significantly lower in villous samples from women with pregnancies complicated by GDM with LGA infants compared to villous samples from normal pregnant women and GDM women with AGA infants. In contrast, the p62 level was significantly higher in placentas from women with GDM and LGA infants than in those from normal pregnancies (e). No differences in the levels of ATG5, beclin-1, LC3B-II, DRAM, and p62 were observed between placentas from women with normal pregnancies and in those from women with GDM and AGA infants. Furthermore, villous samples from GDM women with LGA infants displayed significantly lower *BECN1*, *LC3B*, *DRAM1*, and *p62* mRNA levels than did the samples from women with normal pregnancies (g to j). GDM women with LGA infants also had lower levels of *BECN1*, *LC3B*, and *p62* mRNA than did GDM women with AGA infants (g, h, j). There were no differences in the levels of *ATG5*, *BECN1*, *LC3B*, *DRAM1*, and *p62* mRNA between women with normal pregnancies and the women with GDM and AGA infants. Ten placentas from women with normal pregnancies, 10 placentas from women with GDM and AGA infants, and 10 placentas from women with GDM and LGA infants were used for the analysis. (a to e) Lanes 1–3, villous tissues from normal pregnancies; lanes 4–6, villous tissues from pregnancies complicated by GDM with AGA infants; and lanes 7–9, villous tissues from pregnancies complicated by GDM with LGA infants. (a to j) Horizontal bars represent the median values. *, $P < 0.05$; **, $P < 0.01$, compared to normal pregnant women or GDM women with AGA infants. Normal, normal pregnant women; GDM + AGA, GDM women with AGA infants; GDM + LGA, GDM women with LGA infants; and NS, not significant.

media with 5.6 mM of D-glucose (standard conditions), 5.6 mM of D-glucose and 19.4 mM of L-glucose (hyperosmotic control), or 25 mM of D-glucose (hyperglycemic conditions) for 24 h and the levels of ATG5, beclin-1, LC3B, DRAM, and p62 protein and mRNA were compared. Increasing D-glucose concentration led to significantly lower levels of ATG5, beclin-1, LC3B-II, p62, and DRAM than observed in standard conditions and in the hyperosmotic control (Fig. 4a–e). The transcript levels of these molecules were also lower in cells grown in hyperglycemic conditions than in cells grown in standard conditions or in cells of the hyperosmotic control. However, only differences in *ATG5*, *LC3B*, and *DRAM1* mRNA levels were statistically significant (Fig. 4f, h, and 4j). There was no difference in the protein and mRNA levels of these five autophagy-related molecules in cells cultured under standard conditions and those of the hyperosmotic control.

### 3.6. High glucose concentration is associated with decreased apoptotic changes in cytotrophoblast cells

The effects of increasing glucose concentration on the apoptotic and proliferative changes of cytotrophoblast cells are shown in Fig. 5. Flow cytometry showed that cytotrophoblast cells treated with hyperglycemia had significantly lower percentages of cells at both early (Annexin $V^+$/7-$ADD^-$) and late (Annexin $V^+$/7-$ADD^+$) stages of apoptosis compared to cells grown in standard conditions or cells of the hyperosmotic control (Fig. 5a). Furthermore, the percentages of $M30^+$/Ki-$67^-$ and $M30^-$/Ki-$67^+$ cells were significantly lower and higher, respectively, in cells grown in hyperglycemic conditions than in the other two groups of cells (Fig. 5b). Increasing glucose concentration also led to significantly lower levels of cleaved PARP compared to standard or hyperosmotic control conditions (Fig. 5c). There was no difference in the expression of apoptosis and proliferation markers between cells cultured in standard conditions and those of the hyperosmotic control group.

### 3.7. High glucose concentration modulates the changes of Bcl-2 family of protein expression in cytotrophoblast cells

To further explore the roles of Bcl-2 family members in hyperglycemia-associated apoptotic and autophagic changes in cytotrophoblast cells, we compared the translational and transcriptional levels of Bcl-2, Bcl-xL, Bax, Bak, and Bad among cells of different experimental conditions (standard, hyperosmotic, and hyperglycemic conditions). Cells grown in hyperglycemic conditions had significantly higher levels of Bcl-xL protein and mRNA than did cells grown in standard and hyperosmotic conditions (Fig. 6b and g). In contrast, hyperglycemia significantly reduced Bak protein and mRNA levels compared to cells grown under the other two experimental conditions (Fig. 6d and i). The level of Bad was also decreased in cells grown in hyperglycemic conditions. However, the change in *Bad* mRNA levels did not differ from those of standard condition or hyperosmotic control cells (Fig. 6e and j). There was no difference in the translational or transcriptional levels of Bcl-2 and Bax between the three cell groups (Fig. 6a, c, 6f, and 6h).

## 4. Discussion

Compared to placentas from women with normal pregnancies, we found that placentas from women with GDM and LGA infants were heavier, had fewer autophagic (lower beclin-1 and DRAM levels) and apoptotic (less immunoreactivity of M30 and cleaved PARP) changes,

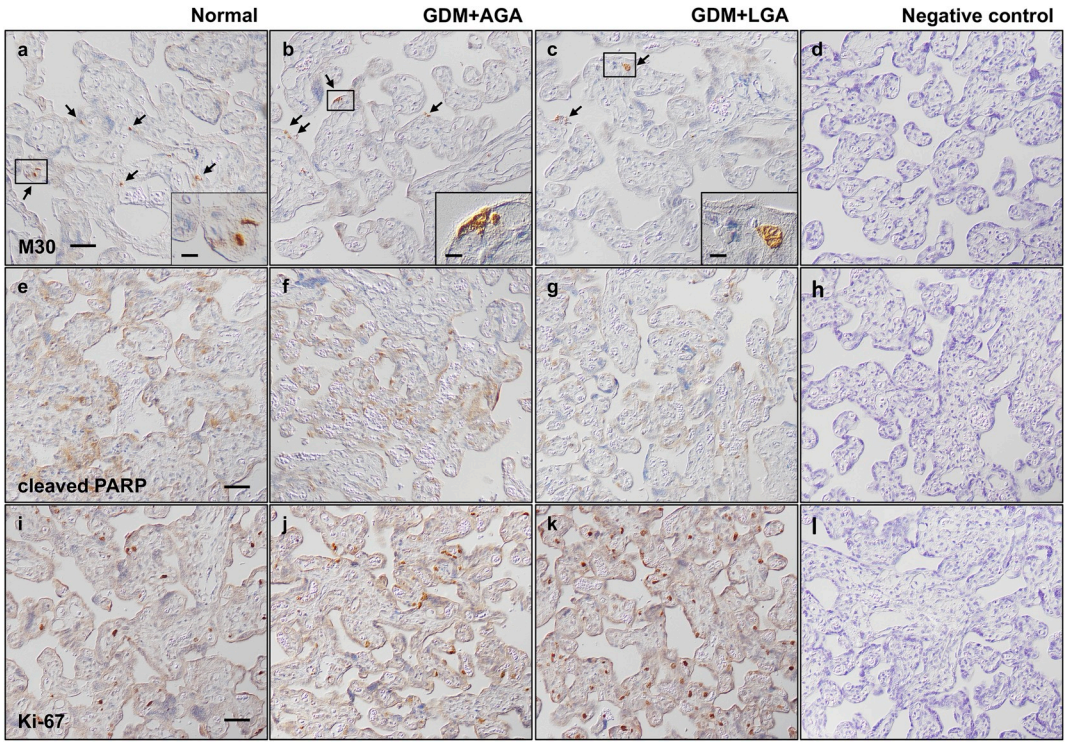


**Fig. 2. Immunohistochemistry for M30, cleaved PARP, and Ki-67 in the placentas from normal pregnancies and those from pregnancies complicated by GDM with AGA or LGA infants.** (a to c) M30 immunostaining was observed in some nuclei in the trophoblast layer (arrows). Compared with normal pregnant women or GDM women with AGA infants, the number of anti-M30 antibody-stained nuclei was lower in GDM women with LGA infants. (e to g) Immunoreactivity of cleaved PARP was noted in focal areas of syncytiotrophoblast and some cytotrophoblasts. The immunostaining of cleaved PARP was less extensive, and the number of anti-cleaved PARP antibody-stained nuclei was lower in GDM women with LGA infants than that in women with normal pregnancies and in GDM women with AGA infants. (i to k) In contrast, villous samples from GDM women with LGA infants showed more cells, including cytotrophoblasts, endothelial cells, and stromal cells, that were positively stained for anti-Ki-67 antibody than villous samples from the other two groups of women. There was essentially no staining at all on the negative controls when the primary antibody was substituted with non-immune IgG (d, h, and l). Scale bar = 50 μm and 10 μm (insets).

**Table 3**
Semi-quantification of the immunostaining intensity of M30 and percentage of cells stained with anti-cleaved PARP or anti-Ki-67 antibodies in villous tissues.

| | Normal pregnancy | GDM with AGA | GDM with LGA | *P* |
|---|---|---|---|---|
| M30 | 1.6 ± 0.5 | 1.5 ± 0.4 | 1.0 ± 0.2[a] | 0.02 |
| Cleaved PARP | 1.2 ± 0.7% | 1.4 ± 0.6% | 0.6 ± 0.3%[a] | <0.01 |
| Ki-67 | 14.8 ± 4.4% | 19.0 ± 4.0% | 28.6 ± 5.7%[b] | <0.001 |

Data presented as mean ± SD.
GDM, gestational diabetes mellitus; AGA, appropriate-for-gestational age fetuses; LGA, large-for-gestational age fetuses.
[a] $P < 0.05$.
[b] $P < 0.001$, compared to women with normal pregnancies.

but had more proliferating cells (increased immunoreactivity of Ki-67). These changes were associated with increased Bcl-xL and decreased Bak levels. There were essentially no differences in the placental levels of autophagy, apoptosis, proliferation, and Bcl-2 family proteins between women with normal pregnancies and those with GDM and AGA infants. Using primary cytotrophoblast cells as a model, we further demonstrated that increasing glucose concentration in the culture medium was associated with decreased autophagic (lower ATG5, beclin-1, LC3B-II, p62, and DRAM levels) and apoptotic (lower percentages of annexin V and M30-positive, cells and lower levels of cleaved PARP) changes compared with the standard culture conditions. Hyperglycemia also led to higher Bcl-xL and lower Bak and Bad levels than did the standard culture conditions. Together, these results indicate that there were differential changes in autophagy and apoptosis between placentas from normal pregnant women and those from GDM women with LGA infants and that some Bcl-2 family proteins are likely involved in the regulation of these changes.

A few studies, with inconsistent results, have evaluated autophagic changes in placentas from women with GDM [19,20]. Ji et al. found significant enhancement of autophagy, reflected by up-regulation of ATG5 mRNA and protein, increased LC3-II, and decreased p62, in placentas from women with GDM compared to those from normal pregnant women [20]. Similar changes were found in HTR8/SVneo cells (an immortalized first-trimester invasive extravillous cell line) treated with a high glucose concentration (30 mM) when compared with cells cultured under physiological glucose concentrations (5 mM) or under hyperosmotic conditions (30 mM mannitol). Avagliano et al. also found that the placental level of LC3-II was higher in women with pregnancies complicated by GDM than in women with normal pregnancies [19]. The level of beclin-1 was lower, and the level of p62 higher, in placentas of women with GDM than in the placentas of normal pregnant women. Furthermore, *LC3* mRNA levels were lower in GDM placentas than in normal placentas. To a certain degree, our findings differ from those of these two previous reports. The discrepancy between ours and previous results is likely caused by differences in GDM diagnosis criteria, in disease severity (with or without LGA infants), modes of delivery (vaginal or cesarean delivery), placental sampling procedures, and cellular types used for *in vitro* experiments (first-trimester cell line or primary cytotrophoblasts from term pregnancy). In this study, we collected placentas from women who underwent elective cesarean deliveries before labor onset to avoid the impact of intermittent placental perfusion, caused by uterine contractions, on the apoptotic and autophagic changes. We also divided our GDM patients into two different phenotypic groups based on neonate birth weight. Using these approaches, we found that there were

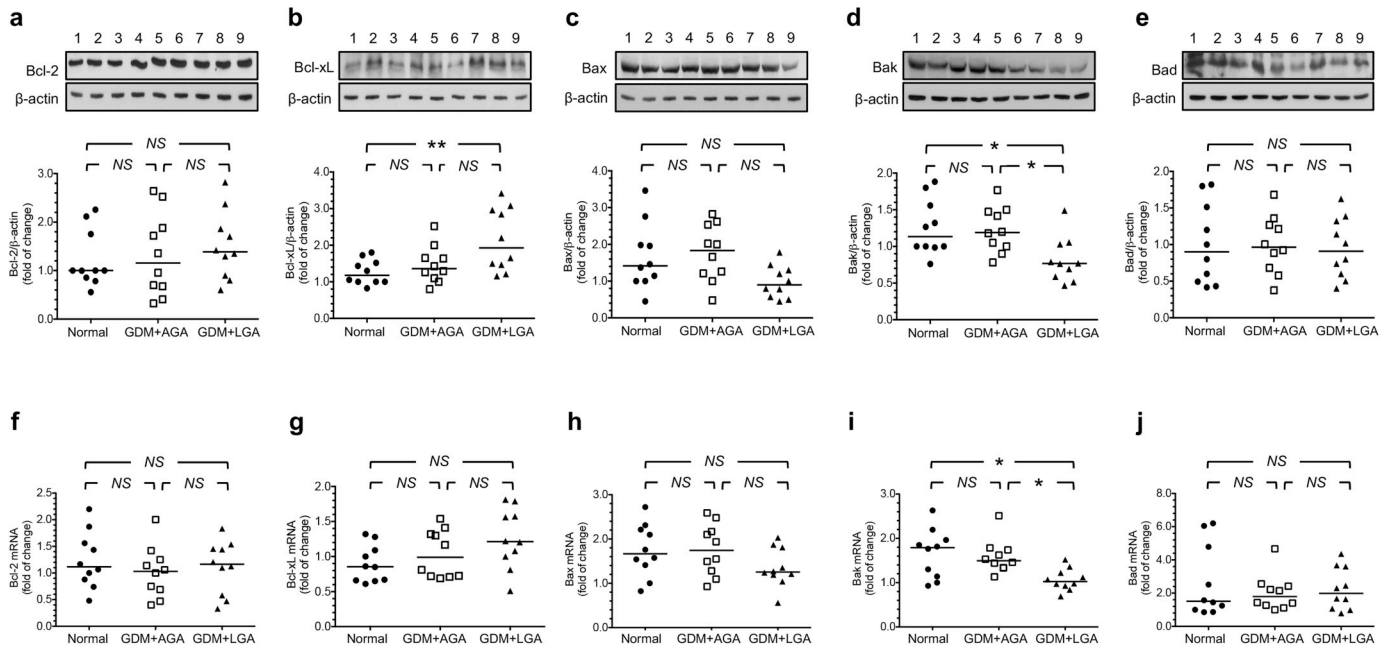


**Fig. 3. Differences in Bcl-2 family protein levels between placentas from normal pregnancies and those from pregnancies complicated by GDM with AGA or LGA infants.** Compared to placentas from normal pregnant women, those from GDM women with LGA infants had a significantly higher level of Bcl-xL (b). However, the levels of Bcl-2 and Bcl-xL did not differ between placentas from normal pregnant women and those from GDM women with AGA infants (a and b). In contrast, placentas from GDM women with LGA infants had significantly lower Bak levels than did those from normal pregnant women and GDM women with AGA infants (d). There was no difference in the levels of Bax and Bad between the three groups of women (c and e). There was no difference in the expression of *Bcl-2* and *Bcl-xL* mRNA between normal pregnant women and women with pregnancies complicated by GDM with either AGA or LGA infants (f and g). GDM women with LGA infants had significantly lower *Bak* mRNA levels than did normal pregnant women and GDM women with AGA infants (i) and no difference was observed in *Bax* and *Bad* mRNA levels between the three groups (h and j). Ten placentas from women with normal pregnancies, 10 placentas from women with GDM and AGA infants, and 10 placentas from women with GDM and LGA infants were used for the analysis. (a to e) Lanes 1–3, villous tissues from normal pregnancies; lanes 4–6, villous tissues from pregnancies complicated by GDM with AGA infants; and lanes 7–9, villous tissues from pregnancies complicated by GDM with LGA infants. (a to j) Horizontal bars represent the median values. *, $P < 0.05$; **, $P < 0.01$, compared to normal pregnant women or GDM women with AGA infants. Normal, normal pregnant women; GDM + AGA, GDM women with AGA infants; GDM + LGA, GDM women with LGA infants; and NS, not significant.

fewer apoptotic and autophagic changes in the placentas from GDM women with LGA infants than in the placentas from normal pregnant women or GDM women with AGA infants. More studies are required to verify our findings and to clarify the relationship between apoptosis, autophagy, and GDM in human placentas.

The causes of differential changes in trophoblast autophagy and apoptosis between placentas from normal pregnant women and placentas from GDM women with LGA infants are unclear. We surmised that increasing glucose concentration could contribute to these changes. GDM women with suboptimal control of blood glucose levels are more likely to have LGA infants or macrosomia, as higher levels of glucose pass through the placenta into the fetal circulation, resulting in hyperinsulinemia [30,31]. This combination of hyperinsulinemia and hyperglycemia likely leads to overgrowth of the fetus [31,32]. Indeed, the rate of macrosomia increases from 20 to 35% when postprandial glucose values increase from an average of 120 mg/dL or less to 160 mg/dL [33]. Furthermore, increasing placental size and placental weight to birth weight ratios are related to the severity of glucose intolerance in GDM women [34]. To test this hypothesis, we cultured primary cytotrophoblast cells in medium with D-glucose levels nearly 5-fold that of standard culture conditions and observed changes similar to our investigation of the placental samples from GDM women with LGA infants. Nevertheless, analysis of insulin or c-peptide levels in the maternal circulation or umbilical cord blood will be quite informative.

Similar to previous reports [14,15], we observed a decrease in apoptotic change in the placentas from GDM women with LGA infants compared to those from normal pregnant women. However, the biological significance of concomitant autophagy and apoptosis decreases in GDM placentas remains unclear. Both apoptosis and autophagy are increased in placentas from women with pregnancies complicated by fetal growth restriction or preeclampsia [10,13,35–41], situations usually associated with small placentas [10,42]. Furthermore, we and others have shown that cytotrophoblast cells treated with hypoxia or oxygen-glucose deprivation have more profound apoptotic changes than do cells cultured under standard conditions, with concomitant increases in autophagic changes [11,43]. These changes suggest an adaptive reaction and a protective effect of autophagy to support cell survival under stress induced by oxygen and/or glucose deprivation. Here, we further demonstrated that there were fewer apoptotic and autophagic changes in the placentas from GDM women with LGA infants than in those from normal pregnant women. Moreover, primary cytotrophoblast cells cultured under increased glucose conditions had fewer apoptotic and autophagic changes than did cells grown under standard conditions. Together, these results provide evidence of the role of autophagy in maintaining trophoblast cellular energy homeostasis during changes in glucose concentration and in eliminating damaged organelles or misfolded proteins in cells committed to die by apoptosis or necrosis [44,45].

Increasing evidence shows that anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, and pro-apoptotic BH3-only proteins, such as Bax, Bak, and Bad, participate in the inhibition and induction of autophagy, respectively [23]. Only a few studies have examined their changes in the placentas of women with GDM. Magee et al. found a significantly higher level of Bcl-2, but not Bak, in GDM placentas than in placentas from normal pregnancies [14]. Cobellis and colleagues found a slight decrease of Bax immunoreactivity in GDM placentas compared to that in normal placentas [16]. In contrast, we found increased Bcl-xL and decreased Bak levels in placentas from GDM women with LGA infants compared with the levels observed in those from women with normal pregnancies. These inconsistencies indicate that further

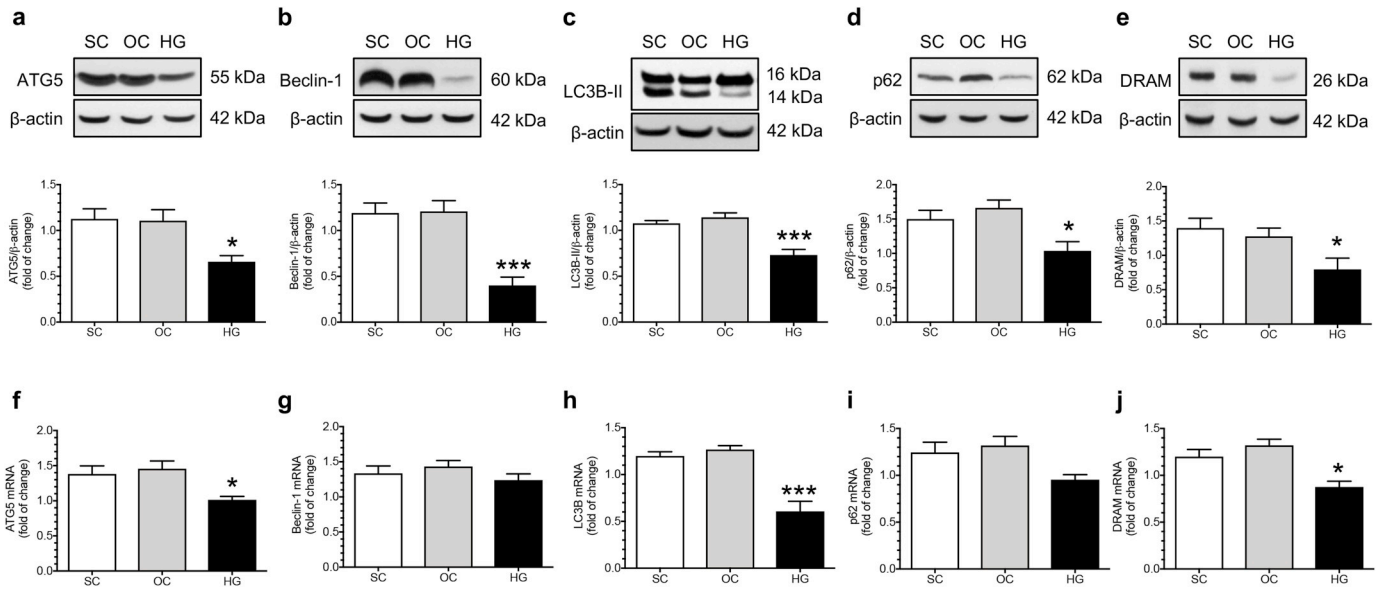


**Fig. 4.** High glucose concentration is associated with decreased autophagic changes in cytotrophoblast cells. **To investigate the effect of increasing glucose concentration on the autophagic changes, primary cytotrophoblast cells were cultured** in media with 5.6 mM of D-glucose (standard conditions), 5.6 mM of D-glucose and 19.4 mM of L-glucose (hyperosmotic control), or 25 mM of D-glucose (hyperglycemic conditions) for 24 h and the levels of ATG5, beclin-1, LC3B, p62, and DRAM protein and mRNA were compared. Increasing D-glucose concentration led to significantly lower levels of ATG5, beclin-1, LC3B-II, p62, and DRAM than observed in standard conditions and in the hyperosmotic control (a to e). The transcript levels of these molecules were also lower in cells grown in hyperglycemic conditions than in cells grown in standard conditions or in cells of the hyperosmotic control. Only differences in *ATG5*, *LC3B*, and *DRAM1* mRNA levels were statistically significant (f, h, j). There was no difference in the protein and mRNA levels of these five autophagy-related molecules in cells cultured under standard conditions and those of the hyperosmotic control. Data are presented as means ± SEM. Six individual experiments were performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared to cells cultured under standard conditions. SC, standard conditions; OC, hyperosmotic control; HG, hyperglycemic conditions.

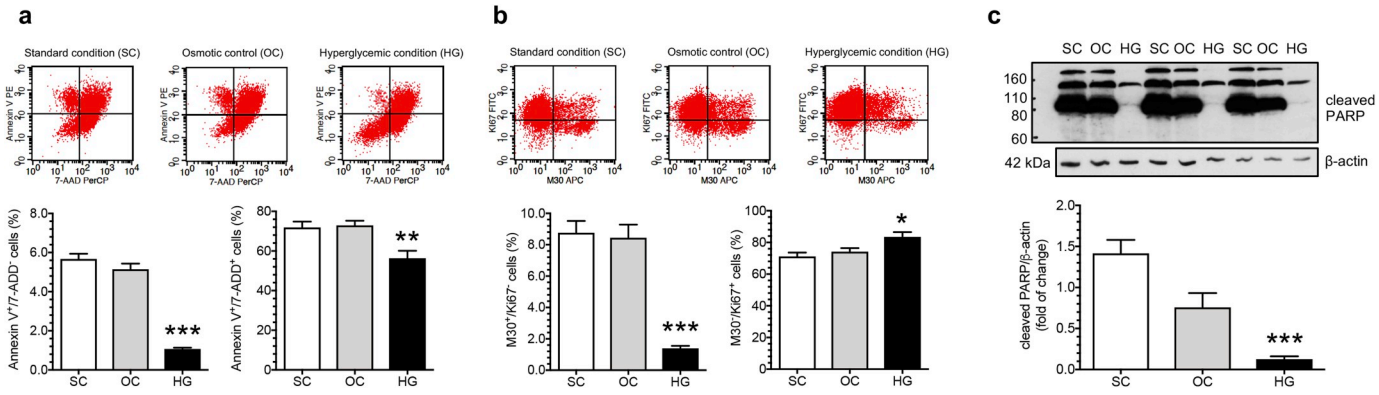


**Fig. 5.** High glucose concentration is associated with decreased apoptotic changes in cytotrophoblast cells. **(a)** Flow cytometry showed that cytotrophoblast cells treated with hyperglycemia had significantly lower percentages of cells at both early (Annexin $V^+$/7-$ADD^-$) and late (Annexin $V^+$/7-$ADD^+$) stages of apoptosis compared to cells grown in standard conditions or cells of the hyperosmotic control. (b) The percentages of $M30^+$/Ki-$67^-$ and $M30^-$/Ki-$67^+$ cells were significantly lower and higher, respectively, in cells grown in hyperglycemic conditions than in the other two groups of cells. (c) Increasing glucose concentration also led to significantly lower levels of cleaved PARP compared to the standard or hyperosmotic conditions. Data are presented as means ± SEM. Six individual experiments were performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared to cells cultured under standard conditions. SC, standard conditions; OC, hyperosmotic control; HG, hyperglycemic conditions.

investigation of the roles of Bcl-2 family proteins in the regulation of autophagy and apoptosis in GDM placentas are warranted.

Several limitations of this study merit attention. First, we found that GDM women tended to have higher systolic blood pressure and pre-pregnancy body mass indexes than normal pregnant women. Given a larger sample size, these differences are likely to be statistically different. It is possible that these factors also contribute to the differences observed in placental markers of autophagy and apoptosis between normal pregnant women and GDM women with and without LGA infants. However, it is difficult to dissect the interactions between maternal obesity, hypertension, and diabetes and study their effects on placental apoptosis and autophagy separately because these diseases share certain common metabolic pathways [46,47]. Similarly, we did not measure the levels of insulin, c-peptide, and other nutrients, such as fatty acids, in the maternal circulation or cord blood. Changes in these factors may also lead to differential changes in autophagy and apoptosis between normal pregnant women and GDM with LGA infants. Second, the use of 25 mM glucose as the hyperglycemic condition may not be similar to physiological conditions, and this concentration is not often observed in women with GDM. However, we used these conditions to study the effects of hyperglycemia on trophoblast apoptosis and autophagy for several reasons. First, this concentration has been widely used to investigate the short-term effects of hyperglycemia *in vitro* on changes in various functions of trophoblast cells from different sources, including

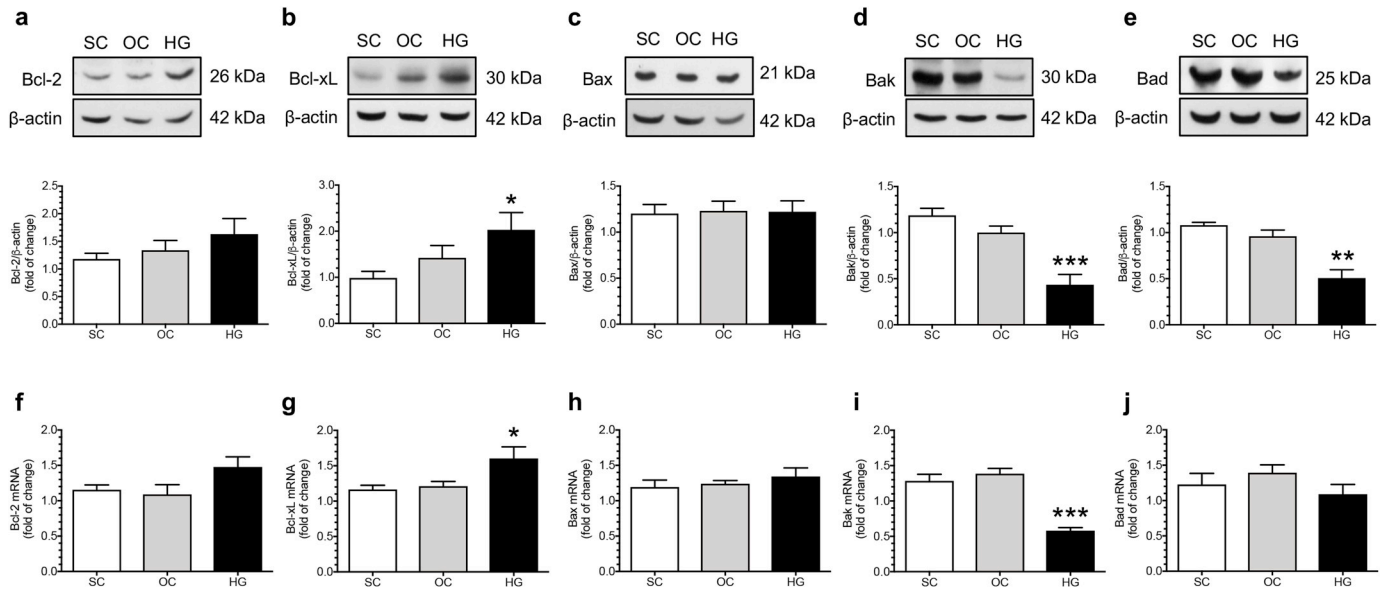


**Fig. 6. High glucose concentration modulates the changes of Bcl-2 family of proteins expression in cytotrophoblast cells.** Cells grown in hyperglycemic conditions had significantly higher levels of Bcl-xL protein and mRNA than did cells grown in standard and hyperosmotic conditions (b and g). In contrast, hyperglycemia significantly reduced Bak protein and mRNA levels compared to cells grown under the other two experimental conditions (d and i). The level of Bad was also decreased in cells grown in hyperglycemic conditions. However, the change in *Bad* mRNA levels did not differ from those of standard condition or hyperosmotic control cells (e and j). There was no difference in the translational or transcriptional levels of Bcl-2 and Bax between these three cell groups (a, c, f, h). Data are presented as means ± SEM. Six individual experiments were performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared to cells cultured under standard conditions. SC, standard conditions; OC, hyperosmotic control; HG, hyperglycemic conditions.

immortal cell lines and cytotrophoblasts isolated from placentas in early or late gestation [48–54]. Additionally, DMEM containing 5.6 or 25 mM glucose is commercially available and ready-to-use. Finally, a recent study by Ji et al. used a glucose concentration of 30 mM to study the effects of hyperglycemia on trophoablast autophagy [20]. We adopted a similar glucose concentration to approach the same question, thus making our findings comparable with their results. Additionally, we did not submit the placentas for pathological examination to exclude the possibility that the observed changes in autophagy and apoptosis were not caused by other pathologies, such as chorangiosis or villitis. Lastly, although we found there were differential changes in autophagy, apoptosis, and Bcl-2 family proteins in the placentas of normal pregnant women and GDM women with or without LGA infants, the changes are not confined to the trophoblasts only and could be observed in other cellular components, such endothelial cells, fibroblasts, and inflammatory cells within the tissue homogenates. Therefore, the results should be carefully interpreted.

In summary, our results show differential changes in autophagy and apoptosis between placentas from normal pregnant women and those from GDM women with LGA infants and that Bcl-2 family proteins are likely involved in the regulation of these changes. Further studies are required to investigate the biological significance of these results and other mechanisms underlying the concomitant decrease of placental apoptosis and autophagy in GDM pregnancies. Understanding the molecular mechanisms contributing to enlarged placentas in women with GDM will inform management and treatment options.

## Declaration of competing interest

None.

## Acknowledgements

This work was supported by grants from the Ministry of Science and Technology, Taiwan (107-2314-B-182A-099 to THH) and Chang Gung Memorial Hospital (CMRP3G0281 and CMRPG1J0071 to THH and CMRPD1G0112 and CMRPD1G0113 to CPW). The authors are grateful to the staff of the Delivery Unit of Taipei Chang Gung Memorial Hospital for their assistance in obtaining the placental materials and to the Genomic Medicine Research Core Laboratory and the Taipei Common Laboratory of Chang Gung Memorial Hospital for providing technical support.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.12.003.

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