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Apoptosis and expression of Bcl-2, Bax, p53, caspase-3, and Fas, Fas ligand in placentas complicated by preeclampsia

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Summary

Aim: To determine whether preeclampsia is associated with an increase in placental apoptosis and altered expression of mediators of apoptosis such as Fas, Fas ligand, caspase-3, p53, Bcl-2 and Bax. *Methods*: Placental samples obtained from 20 women with preeclampsia and 14 normal women were analyzed using the Klenow, Frag-EL DNA fragmentation detection kit for apoptosis. Expression of Fas, Fas ligand, caspase-3, p 53 Bcl-2, and Bax was assessed using immunohistochemistry. *Results*: The median percentage of apoptotic nuclei in trophoblasts was significantly higher for the preeclampsia group than for the controls (6.9 vs 0.25; p =.001). Fas ligand expression was significantly higher in the decidua among the subjects with preeclampsia compared with controls (1.2 vs 0; p = .023). Otherwise no difference was observed in the expression of Bax , Bcl-2, p53, caspase-3, and Fas between groups. *Conclusion*: Increased apoptosis in preeclampsia may not be associated with significant alterations in Fas, Fas ligand, caspase-3, p53, Bcl-2 and Bax expression.

Key words: Apoptosis; Placenta; Preeclampsia.

Introduction

Apoptosis, a form of programmed cell death, is an essential physiologic process required for normal development and maintenance of tissue homeostasis. Apoptosis is an energy-dependent cell death where no materials remain in the extracellular space after phagocytosis and thereby no immune response occurs [1]. The other form of programmed cell death is autophagy which consists of elements of both apoptosis and necrosis [2]. In contrast to apoptosis, necrosis does not require cellular energy and results in the loss of cytoplasm and organelles to the extracellular matrix [3].

Preeclampsia is a major cause of maternal and perinatal mortality and morbidity worldwide [4]. It has been reported that preeclampsia is associated with increased apoptosis in villous trophoblasts [5-7].

Apoptosis may result from extrinsic and intrinsic pathways. The former is started with an external signal and mediated by interactions of membrane receptors and ligands, such as Fas, Fas ligand, and tumor necrosis factor (TNF)-α. The latter is initiated within the cell and involves alterations of mitochondrial membrane permeability. These are not distinct pathways and cross-activation occurs [8]. Both pathways join in the activation of aspartate-specific cystine proteases (caspases). The aspartate-specific cysteine protease (caspase) cascade is now believed to be the main pathway by which cellular death is managed. The most prevalent caspase in the cell is caspase-3 which is predominantly expressed in syncytiotrophoblast cytoplasm [3, 9].

Members of the BCL-2 family regulate the intrinsic pathway of apoptosis via effecting mitochondrial membrane permeability. BCL-2, an anti-apoptotic member of the BCL-2 family, is inversely proportional to apoptosis and has been shown in syncytiotrophoblast cytoplasm throughout pregnancy [7, 9]. Bax, a pro-apoptotic member of the Bcl-2 family, has been shown in isolated areas of cytotrophoblasts and syncytiotrophoblasts [10, 11].

p53 is a sequence-specific transcription factor capable of inducing cell growth arrest or apoptosis[12]. In normal pregnancy, p53 is predominantly expressed in cytotrophoblast nuclei and increased expression is found in proliferative areas [13].

There are conflicting results regarding apoptosis and expression of regulators in placenta complicated by preeclampsia. Apoptosis is increased in trophoblasts in preeclampsia, whereas alterations in expression of regulator proteins do not show consistency with the cascade [5, 7]. We conducted a study to determine whether preeclampsia is associated with apoptosis and the expression of Fas, Fas ligand, p53, caspase-3 Bcl-2, and Bax.

Materials and Methods

Twenty placentas from pregnancies complicated with preeclampsia and 14 placentas from controls were collected between 2001 and 2003 at Akdeniz University, School of Medicine, Department of Obstetrics and Gynecology. The study was approved by the review board of Akdeniz University. All women were greater than 20 weeks' gestation at delivery. Samples were collected after vaginal or cesarean delivery. Preeclampsia was defined as systolic blood pressure (BP) greater than 140 or diastolic BP greater than 90, with proteinuria on a catheterized urine specimen of at least 1+. Small for gesta-

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tional age was defined as weighing less than the 10th percentile of birth weight for the delivery week [14, 15]. Preterm delivery defined as birth before 37 weeks of gestation. Control subjects had BPs less than 140/90 Hg/mm, lacked proteinuria on dipstick, and had no evidence of chronic hypertension, preeclampsia, intrauterine growth restriction, gestational hypertension or any disease that alters placental function.

The placental tissues obtained were immediately fixed after delivery in 4% buffered neutral formaldehyde solution, dehydrated, and embedded in paraffin. Sections of 4 μm were deparaffinized in accordance with standard histologic techniques. Immunohistochemical staining was performed by using the avidin/biotin immunoperoxidase method with the use of a polyvalent immunoperoxidase. After washing with phosphate-buffered saline solution, the sections were immersed in 3% hydrogen peroxide to inhibit endogenous peroxidase activity. Specimens were incubated with the antibodies of: Caspase3 (ms 1123-7, 3CSPO3, prediluted), Fas (ms1098-r7, 95C03, prediluted), Fas ligand (ms1108-ry, FSL01, CA), Bax (ms711-ry, 2D2, 1/25 diluted), Bcl-2 (ms123-ry, 100/D5, prediluted), p53 (ms738-ry, DO-7, 1/50 diluted), (Neomarkers Union CA). The first incubation was done with the primary antibody and the second incubation with biotinylated polyvalent antibody. The third incubation was carried out with avidin-horseradish peroxidase. Thereafter, a chromogenic reaction was developed by use of incubation with a freshly prepared solution of 3-amino-9-ethylcarbazole and hydrogen peroxide. The sections were counterstained with Harris hematoxylin, mounted in glycerine phosphate buffer solution, and examined microscopically. The semiquantitative immunohistochemical scoring system (HSCORE) was calculated using the equation:

$$\text{HSCORE} = \sum \text{Pi}\ (\text{i}+1)$$

where i = intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively), and Pi as the percentage of stained trophoblasts, stroma, or decidua of each intensity. Previous studies that used the HSCORE have determined that the technique yields low inter- and intraobserver variation and is a suitable semiquantitative method for comparing immunostaining results [5]. More than 1000 cells were counted in each sample.

Klenow (Frag-EL DNA fragmentation detection kit, (Cat #QIA21, Oncogene, San Diego, CA) was used to detect the apoptotic index. Fifteen high power field was evaluated and 3000 cells were counted for each patient. The apoptotic index is defined as the percentage of cells that were stained by the Klenow method divided by the total number of cells counted.

Statistical comparison was done with Mann-Whitney U and chi-square tests. A p value of $< .05$ was significant. Statistical analysis was performed by using SPSS 10 (SPSS Inc., Chicago, IL).

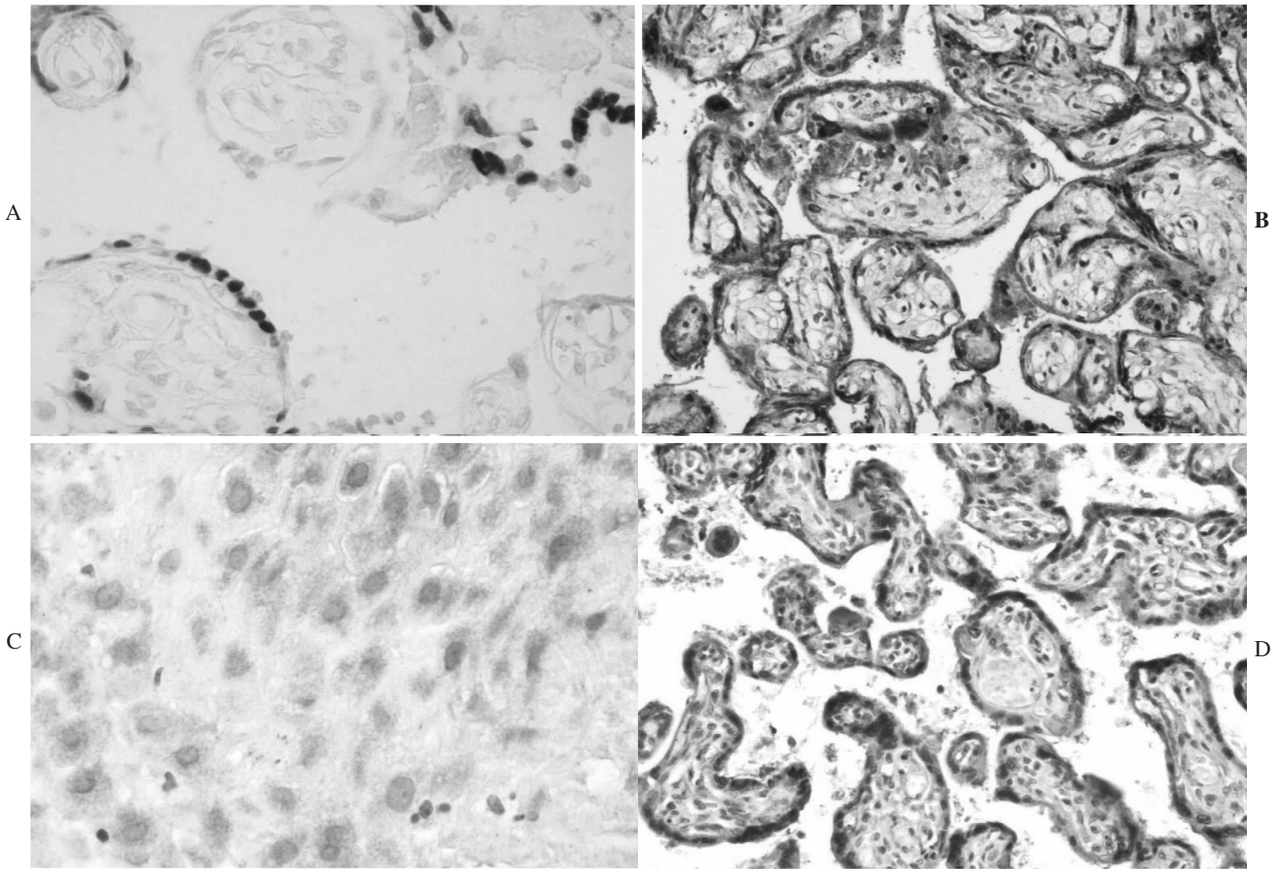

Figure 1. — Apoptosis in sections of preeclamptic placenta assessed by the FragEL-Klenow DNA fragmentation detection method (original magnification x 400) (A); Immunostaining for Fas (original magnification x 200) (B); Fas ligand (original magnification x 200) (C), and caspase 3 (original magnification x 400) (D) in placentas with preeclampsia.

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Results

Demographic characteristics are shown in Table 1. Two pregnancies in the preeclampsia group were twins. Fourteen cases in the preeclampsia group were defined as severe and seven patients had eclamptic convulsions. Small for gestational age was diagnosed in 11 pregnancies with preeclampsia. Thirteen patients in the preeclampsia group received magnesium sulfate for convulsion prophylaxis. Only five patients delivered below 37 gestational weeks in the control group. No apoptosis was detected in seven cases (6 controls and 1 preeclampsia). Stromal apoptosis was detected only in eight cases with preeclampsia and two cases from the controls. Nonstained cases were defined as negative in statistical analysis. Median percent apoptotic nuclei was significantly higher in villus trophoblasts in the preeclampsia group than in the control group (6.9 vs 0.25; $p = 0.001$) (Figure 1). No statistically significant difference was observed in apoptotic nuclei in stroma beween groups. Expression of apoptotic, anti-apoptotic proteins (Figure 1) and percentage of cases positively stained are shown in Table 2. Fas ligand expression in decidua was significantly higher in the preeclampsia group than the control group. p53 and Bax expression was detected in a very small number of patients in both groups. Among preeclamptic cases, there was no statistically significant difference in terms of apoptosis in trophoblast and villous stroma between cases with eclampsia versus without eclampsia and cases with small for gestational age and normal growth.

Discussion

In our study trophoblast apoptosis was significantly higher in placentas with preeclampsia than placentas without pathology. This finding is consistent with the literature [5-7].

Hypoxia and hypoxia-reoxygenation injury can induce apoptosis in cultured trophoblasts [16, 17]. Preeclampsia is characterized by abnormal trophoblast invasion to spiral arteries of decidua and myometrium which leads to inadequate uteroplacental blood flow and thereby causing fetal compromise. Hypoxia due to inadequate trophoblast invasion can lead to increased apoptosis in the placenta.

Table 1. — *Demographics*.

	Preeclampsia (n = 20)	Control (n = 14)	P
Age (yrs)	25 (9)	31 (9)	NS
Gravidity (n)	2 (2)	2 (2)	NS
Parity (n)	1 (1)	0 (1)	NS
Abortion (n)	0 (1)	0 (1)	NS
CS	19 (95%)	5 (37%)	.001
Gestational age (wks)	35 (5)	38 (5)	NS
Preterm birth (n)	14 (70%)	5 (35.7%)	.048
Birth weight (g)	1840 (1025)	2810 (1500)	.018

Data are presented as median (interquartile range) or number (percentage). CS 0 cesarean section. NS = Non significant

Table 2. — *Expression of mediators of apoptosis in placental samples*.

	Preeclampsia HSCORE n = 20		Control HSCORE n = 14		*p*
BCL-2		% of stained cases		% of stained cases	
Tropholast	2.8 (0.7)	100	2.1 (1.6)	100	NS
Stroma	0.6 (0.9)	85	0.75 (1.275)	64	NS
Decidua	1.6 (2.15)	85	1.2 (1.25)	78	NS
p 53					
Trophoblast	0 (0.375)	25	0 (0.2)	21	NS
Stroma	0 (0	20	0 (1.525)	42	NS
Fas					
Trophoblast	2.8 (1.9)	100	3.2 (0.9)	100	NS
Stroma	0.6 (0.975)	55	0.85 (1.525)	57	NS
Decidua	2.8 (1.9)	90	2 (2.15)	85	NS
Fas ligand					
Trophoblast	0.3 (1.625)	75	0.1 (0.5)	50	NS
Decidua	1.2 (2.075)	75	0 (0.55)	50	0.023
Caspase 3					
Trophoblast	2.8 (2.775)	95	1.55 (1.875)	92	NS
Decidua	0.4 (1.8)	55	0 (0.2)	28	NS
Bax					
Decidua	0 (0.15)	25	0 (0.125)	42	NS

Data are presented as median (interquartile range). NS = Non significant

Apoptosis in preeclampsia and intrauterine growth restriction (IUGR) will disrupt normal cytotrophoblast cell turnover in the villus, preventing the normal replenishment of mRNA for the syncytiotrophoblast, which may decrease placental function [3].

Preeclampsia is associated with increased shedding of syncytiotrophoblast microparticles (STMB) into the maternal circulation [18, 19]. STMB can cause endothelial cell dysfunction which is the main event in preeclampsia leading to maternal response [20, 21]. Recently it has been shown that shed trophoblasts are predominantly apoptotic in an in-vitro model [22]. Increased apoptosis followed by increased shedding of STMB may play a role in maternal response in preeclampsia. Although the data is scarce, there may be a possible link between increased apoptosis in preeclampsia and maternal complications.

Although increased trophoblast apoptosis in preeclampsia is well established, the precise role of regulator proteins is yet to be determined. There are conflicting results regarding the expression of regulator proteins in preeclampsia using immunohistochemistry, Western blotting or both. The Bcl-2 family has a role in the intrinsic apoptosis pathway via controlling the mitochondrial permeability. It was reported that preeclampsia and IUGR are associated with decreased Bcl-2 expression, although another study showed no difference in preeclampsia and IUGR as in our study [5, 23]. No alteration has been reported in Bax expression in placentas complicated by preeclampsia and IUGR [5, 23]. In our study, Bax expression was detected in a very small number of cases and

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gave no information in terms of programmed cell death. Similarly, in some other studies Bax expression was found to be present in isolated areas of cytotrophoblast and syncytiotrophoblast cytoplasm [10, 11].

Increased p53 expression has been reported in villous trophoblasts with IUGR [23]. In contrast, another study found down-regulation of p53 expression in placentas with IUGR, and no difference in preeclampsia and up-regulation in HELLP syndrome [24]. In our study, p53 expression was very low. The activity of p53 is regulated by Mdm2 which inhibits the transcriptional activity of p53 and, more importantly, promotes its degradation by the proteasome [12]. Imbalance between p53-Mdm2 and Bax/BCL-2 at any point may be sufficient to induce apoptosis without significant differences in expression of these proteins [3].

Many caspases have been shown in villous trophoblasts, however caspase-3 is the most investigated one due to its main role in cell digestion [3]. Active caspase-3 expression has been demonstrated mainly in normal villous syncytiotrophoblasts and increased in placentas with IUGR [25]. However in our study no significantly different alteration was observed in active caspase-3 expression. Caspase-3 activity is regulated by the balance of inhibitor proteins. Smac, a mitochondrial protein, binds these proteins preventing their inhibitory action which is significantly elevated in villous trophoblasts in preeclampsia [26]. Imbalance between regulator proteins rather than significant increase in caspase-3 expression may lead to apoptosis in preeclampsia.

The Fas/Fas ligand system is one of the main apoptotic pathways which belongs to the TNF-α/TNF-α receptor family. The binding of the Fas receptor by Fas ligand leads to downstream activation of a cascade of intracellular proteolytic enzymes ending in apoptosis [27]. It has been shown that Fas expression and Fas ligand expression were significantly decreased in trophoblasts obtained from placentas with preeclampsia compared with controls [5]. In contrast, in another study no difference was found in the intensity of Fas immunostaining in syncytiotrophoblasts in placentas complicated by severe preeclampsia or IUGR [7]. In our study, in placentas with preeclampsia decidual fas ligand expression was significantly greater than controls. This may be a sign of impaired apoptosis in neutrophils in preeclampsia contributing to maternal response [28].

Although apoptosis is increased in trophoblasts, the activated pathway is still unclear. According to alterations in regulator proteins from intrinsic or extrinsic pathways, both ways seems to be activated in preeclampsia [5,7] . However more research needs to document the exact pathways in apoptosis in placentas complicated by preeclampsia.

Preeclampsia has also been subclassified as early and late onset [29]. Both have different etiologies and clinical expressions. Early-onset disease (< 34 + 0 weeks) is characterized with abnormal trophoblast invasion of maternal spiral arteries, abnormal uterine and umbilical artery Doppler and intrauterine growth restriction. Late onset preeclampsia (≥ 34 + 0 weeks) is associated with normal or slightly altered trophoblast invasion of maternal spiral arteries, no or slight changes in uterine and umbilical artery Doppler and normal fetal growth [30]. In our study subanalysis of placentas with preeclampsia in terms of apoptosis in trophoblasts and decidua between cases with small for gestational age and cases with normal weight revealed no difference. It seems that both forms are characterized with increased apoptosis in trophoblasts.

In our study there was no statistically significant difference between the gestational weeks at delivery between groups. However, most of the deliveries were close to term in the control group. Apoptosis has been shown to increase in normal placentas throughout pregnancy [31]. Despite this normal increase in programmed in cell death in uncomplicated placentas, placentas complicated with preeclampsia showed significant increase in apoptosis.

The FragEL-Klenow DNA fragmentation detection kit and terminal dUTP nick-end labelling (TUNEL) kit are both enzymatic detection methods and were applied to the tissues to show nuclei of cells with DNA fragmentation. The Klenow kit has the advantage that it counterstains "healthy" nuclei within the same procedure. This kit is also able to detect both single- and double-strand DNA breaks [32].

In conclusion increased apoptosis in preeclampsia may be associated with imbalance between effector and inhibitor regulator proteins of the cascade rather than increased expression of a single protein. Further research is needed to assess the role of regulator proteins in preeclampsia. Understanding the exact roles of proteins may lead to development of therapeutic options to reverse the cascade.

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