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Expression of Nuclear Factor-Kappa B and Placental Apoptosis in Pregnancies Complicated with Intrauterine Growth Restriction and Preeclampsia: An Immunohistochemical Study

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ABAN, M., CINEL, L., ARSLAN, M., DILEK, U., KAPLANOGLU, M., ARPACI, R. and DILEK, S. *Expression of Nuclear Factor-Kappa B and Placental Apoptosis in Pregnancies Complicated with Intrauterine Growth Restriction and Preeclampsia: An Immunohistochemical Study.* Tohoku J. Exp. Med., 2004, **204** (3), 195-202 —— Preeclampsia affects 7-10% of all pregnancies, and is a major cause of maternal and fetal morbidity and mortality. Although enhanced apoptosis is well known in placentas with preeclampsia, the role of transcription factor nuclear factor-kappa B (NF-κB) in the process is still being debated. In this work, we investigate the relationship between NF-κB expression and trophoblastic cell apoptosis in pregnancies complicated with preeclampsia or intrauterine growth restriction (IUGR) by immunohistochemical analysis of NF-κB and three apoptosis related markers: bcl-2, caspase-3, and M30 CytoDeath antibody that identifies early apoptotic changes in the cytoskeleton related to action of caspase. The study was conducted on placental samples from 19 preeclamptic, 5 IUGR-complicated and 10 normal pregnant women. The three conclusions from the statistical analysis of the data are obtained; (i) Significantly higher expression of NF-κB in IUGR-complicated ($p = 0.003$) and preeclamptic placentas ($p = 0.004$) than the control placentas, (ii) significantly higher M30 index and caspase 3 expression in IUGR and preeclampsia placentas ($p = 0.003$), and (iii) decreased expression of bcl-2 in IUGR and preeclampsia placentas ($p = 0.001$). Based on these observations, we suggest that increased trophoblastic apoptosis is at least partially induced by NF-κB and reduced bcl-2 expression. ——— preeclampsia; IUGR; apoptosis; NF-κB; bcl-2

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Preeclampsia affects 7-10% of all pregnancies, and is a major cause of maternal and fetal morbidity and mortality. Despite recent advances in medicine, its etiology still remains unknown (Roberts et al. 1990). Maintenance of trophoblast structure and specialized function is essential for the provision of adequate oxygen and nutrients to the fetus. Fetal complications of preeclampsia and intrauterine growth restriction (IUGR) are commonly attributed to clinical conditions that lead to underperfusion and hypoxia of the placenta (Levy et al. 2002). The pathogenic mechanisms of preeclampsia converge on the vascular endothelium and numerous stimuli, including oxidative stress. Furthermore, TNF-α and high glucose are well known to induce apoptosis in endothelial cells (Muschel et al. 1995; Haimovitz-Friedman 1997).

Apoptosis is a form of programmed cell death and has been observed in placentas of normal human pregnancies (Smith et al. 1997a, b; Allaire et al. 2000). There are some studies that have focused on the role of apoptosis in placenta in relation to the pathophysiology of preeclampsia and IUGR (DiFederico et al. 1999; Levy et al. 2002). It is well known that placentas from women complicated with preeclampsia or IUGR show enhanced apoptosis as compared to placentas of normal pregnant women (Smith et al. 1997b; Allaire et al. 2000; Ishihara et al. 2002). However, the molecular mechanisms leading to apoptosis are complex, and include an ever-expanding list of signaling molecules such as the bcl-2 family of genes, the caspase-3 and NF-κB (Hetts 1998; Adams and Cory 1999).

The balance between pro- and anti-apoptotic proteins determines whether apoptosis will be triggered or not. Caspase-3 is a cysteine protease protein that exists as inactive zymogene in almost all cells, and is involved in the development of apoptotic cell death. Activation of caspase-3 occurs in response to a variety of apoptotic inducers (Mallat et al. 1997). It has been shown that cytokeratins, in particular cytokeratin 18, which is cleaved by caspase 3 or 7, are affected in the early stages of apoptosis (Leers et al. 1999). M30 CytoDeath® antibody recognizes a caspase-mediated fragment of cytokeratin 18 and therefore is most likely found only in caspase-dependent apoptosis, but not in nonapoptotic cells (Kadyrov et al. 2001). On the other hand, one of the major genes responsible for regulating apoptotic cell death is the protooncogene, bcl-2, which can prevent apoptosis through regulation of cellular antioxidant defense mechanisms (Hockenbery et al. 1993).

NF-κB is a redox-sensitive transcription factor regulating a battery of inflammatory genes, and has a variety of different effects in numerous pathological states. Although the role of NF-κB in programmed cell death is still controversial, some researchers have claimed a direct action of NF-κB in the process of apoptosis (Kitajima et al. 1996; Qin et al. 1998; Matsushita et al. 2000). It has also been shown that the activation of NF-κB binding and increased caspase-3 both affect the endothelial cells under hypoxic conditions (Roebuck et al. 1999). However, little is known about the relationship between the expression of NF-κB and apoptotic changes in trophoblastic cells of IUGR and preeclampsia in humans. In this study, we investigate NF-κB expression and placental trophoblastic apoptosis and its relation with bcl-2 in placental specimens that have been obtained from pregrant women complicated with preeclampsia or IUGR.

Materials and Methods

Placental materials

This study was conducted on 34 pregnant women between January 2001 and December 2003 in the Department of Obstetrics and Gynecology at the University of Mersin. Written informed consent was obtained from all subjects, and the study protocol was approved by Institutional Ethics Committee. Ten of the placentas were considered between 36 and 38 weeks of singleton pregrancy and were from normal patients who had cesarean delivery for various obstetric indications such as previous cesarean section and pre-

sentation anomalies. All normal term infants had birth weights ≧10th percentile of the individualized birth weight ratio. Term placentas complicated by either severe preeclampsia or IUGR from a singleton pregnancy between 36 and 38 weeks of gestation were obtained from 24 patients who had cesarean delivery. Severe preeclampsia was defined by the criteria established by the American Collage of Obstetric and Gynecology (ACOG 1996 Technical bulletin no: 219).

The IUGR was based on 3 criteria: ultrasonographic evidence of deviation from an appropriate growth percentile, clinical evidence of suboptimal growth, and individualized birth weight ratios of < 10th percentile. None of the women had smoking or drinking habits, or had a concurrent medical illness. The gestational age of the placenta was first determined by an approximate computation of the duration of pregnancy from patient's last menstrual period and was further confirmed with ultrasonography.

Histopathological material

Villous tissues from 3-5 cotyledons were dissected, rinsed in saline solution, and fixed with %10 formalin for 24 hours at 4°C. Hematoxylin and eosin stained sections from each specimen were examined under light microscope and representative sections from each tissue sample were selected for immunohistochemistry.

Immunohistochemistry for NF-κB, bcl-2, M30 (CytoDeath monoclonal antibody, 1:50, Roche®) and Caspase-3 was performed using a combination of the streptavidin-biotin-peroxidase method and microwave antigen retrieval on formaline-fixed paraffin-embedded tissues. After deparaffinization, sections were treated with 10% hydrogen peroxidase in filtered water to block endogenous peroxidase activity. To retrieve antigen, slides were treated with 10 mmol/liter citrate buffer (pH = 6.8) for 10 min. After preincubation with Ultra V block (LabVision®) for 20 minutes, sections were incubated with primary antibody for an hour at room temperature for bcl-2 (monoclonal mouse anti-Human bcl-2 oncoprotein, 1:50, Dako®), NF-κB (Rabbit polyclonal antibody, p50 Ab-2, 1:75, Neomarkers®), M30 or caspase-3 (Rabbit polyclonal antibody, CPP32, 1:50, Neomarkers®) followed sequentially by biotinylated goat anti-polyvalent (Lab Vision®) for 20 minutes, and streptavidin peroxidase complex (Lab Vision®) for 30 minutes. 3-Amino-9 Ethylcarbazole/AEC (Lab Vision®) and hematoxylin were used as chromagen and for nuclear counterstain, respectively. Negative control was achieved by omitting the primary antibody. The positive controls were tonsil for bcl-2, testis for NF-κB, infiltrative ductal carcinoma of breast for caspase-3 and M30.

Scoring and statistical analysis

Immunohistochemical evaluation was carried out only in the epithelial component of the trophoblastic cells. All specimens were examined by the pathologist (CL) who was blinded to clinical conditions. The staining score for bcl-2, caspase-3, or NF-κB was labeled as "0" for no immunostaining, "1+" for weak and focal immunostaining, "2+" for weak and diffuse immunostaining, "3+" for strong and diffuse immunostaining according to the overall staining feature. Previously defined (Chiu et al. 2001) M30 index was used, whith is the percentage of positively stained area to the total area of villous trophoblast in each section. The sections were examined at high power (× 400). Kruskal-Wallis test was used to analyse the relationship between scores from each experimental groups.

Results

Table 1 summarises the clinical characteristics for each patient. Immunohistochemical analysis of the NF-κB, bcl-2, or caspase-3 expressions and the M30 index are shown in Table 2. All cases showed positive staining immunolocalized to the cytoplasm of trophoblastic cells (Figs. 1-4). In IUGR and preeclampsia groups, apoptosis assessed by M30 CytoDeath® was observed to be more significant than in the control group (p =

0.001). Sixty percent of the control group had 3+ staining score with bcl-2 and no case was found with 1+ staining score in the control group. In IUGR and preeclampsia groups, there were 1+ staining scores of 60% and 57.9%, respectively. On the other hand, 3+ staining score was observed only in 10.5% of the patients in preeclampsia group, but none in the IUGR group.

The other important observations from the statistical analysis of the data can be summarized as follows: (i) significantly higher staining score with bcl-2 in the control group ($p = 0.001$), (ii) significantly lower staining scores with caspase-3 and NF-κB in the control group than in IUGR ($p = 0.003$) and preeclampsia groups ($p = 0.004$), (iii) 1+ caspase staining in 80% of the patients in the

TABLE 1. *The clinical characteristics of each group*[*]

	Control $n = 10$	Preeclampsia $n = 19$	IUGR $n = 5$	p value
Age (yrs)	28.2 ± 3.3	25.8 ± 5.6	27.2 ± 1.9	> .05
Gestational age (wks)	37.4 ± 0.8	36.4 ± 0.5	37.0 ± 1.0	> .05
Fetal weight (grs)	3250 ± 155	3089 ± 204	1890 ± 354[**]	< .05

[*] Data were shown as means and standard deviations.
[**] Significantly different from other groups.

TABLE 2. *The descriptive statistical analysis of expression scores of caspase 3, Bcl-2, NF-κB and M30 index in three groups*[*]

	Control $n = 10$	Preeclampsia $n = 19$	IUGR $n = 5$	p value
Caspase 3	1.2 ± 0.42 (10.10)[**]	1.95 ± 0.78 (19.03)	2.6 ± 0.55 (26.50)	0.003
Bcl-2	2.6 ± 0.52 (26.50)[**]	1.53 ± 0.70 (14.03)	1.40 ± 0.55 (12.70)	0.001
NF-κB	1.40 ± 0.52 (9.60)[**]	2.26 ± 0.73 (19.95)	2.60 ± 0.55 (24.0)	0.004
M30	6 ± 5.55 (7.55)[**]	21.5 ± 15.86 (21.19)	24.8 ± 21.15 (20.8)	0.001

[*] Data were shown as means and standard deviations and mean ranks in parentheses.
[**] Significantly different from other groups.

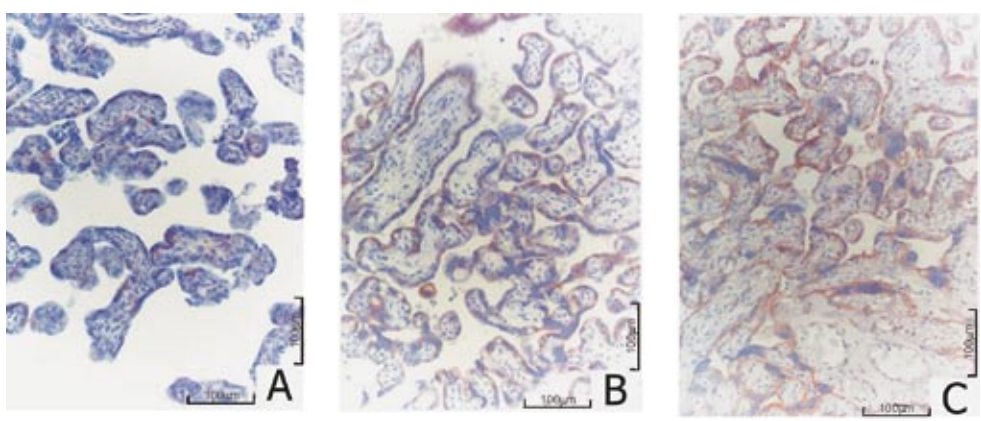

Fig. 1. Cytoplasmic staining with Caspase-3 in placentas. A, Control; B, IUGR; C, Pre-eclampsia. Immunohistochemical staining ×200 (Brown color indicates positive staining for Caspase-3).

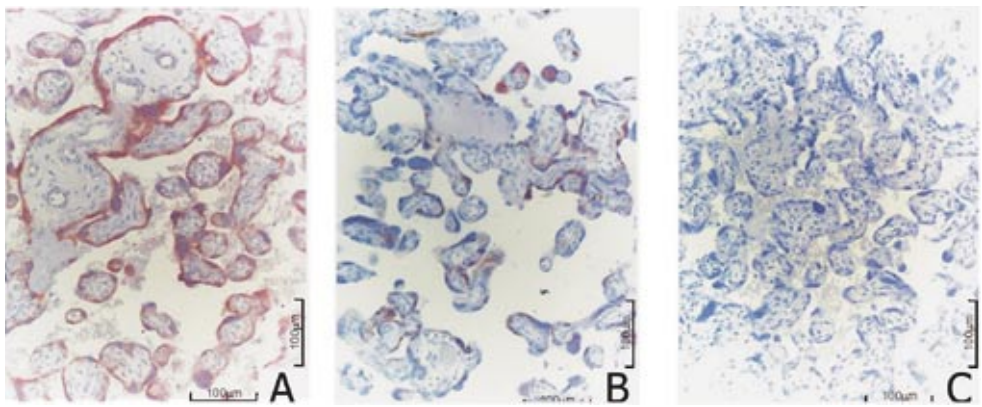

Fig. 2. Cytoplasmic staining with bcl-2 in placentas. A, Control; B, IUGR; C, Preeclampsia. Immunohistochemical staining × 200 (Brown color indicates positive staining for bcl-2).

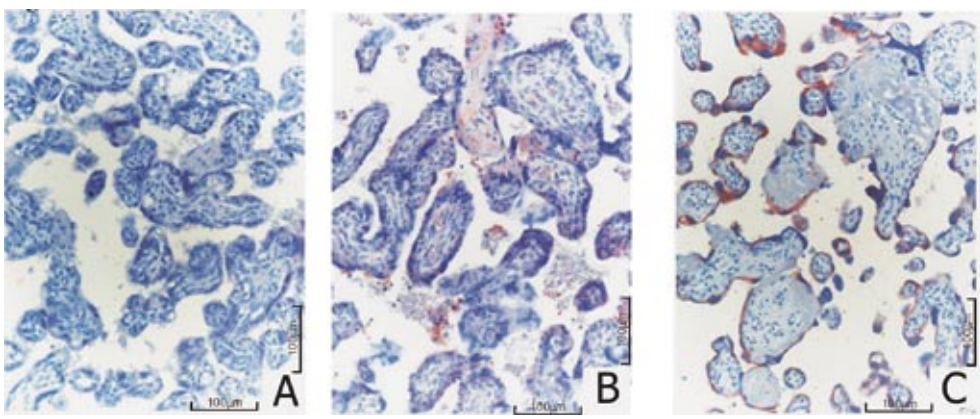

Fig. 3. Cytoplasmic staining with NF-κB in placentas. A, Control; B, IUGR; C, Preeclampsia. Immunohistochemical staining × 200 (Brown color indicates positive staining for NF kappa B).

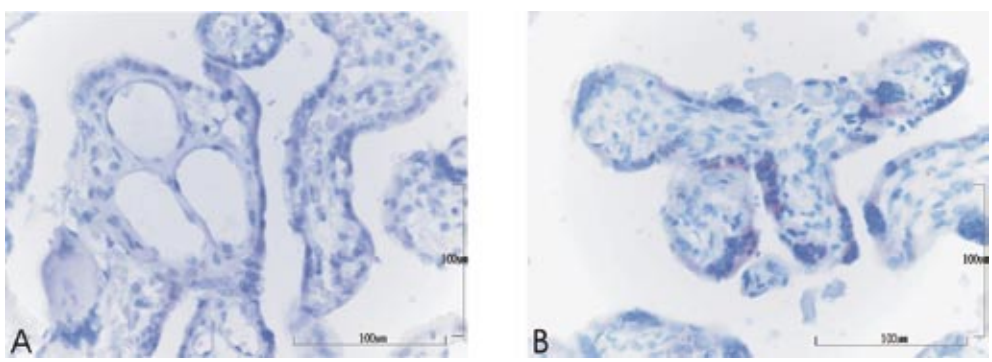

Fig. 4. Photomicrograph of cytoplasmic staining with M30 CytoDeath indicating apoptosis in placenta. A, Control; B, preeclampsia. Immunohistochemical staining × 400 (Brown color indicates positive staining for M30).

control group, (iv) 3+ NF-κB staining scores in 0%, 60% and 42% of the patients in the control, IUGR and preeclampsia groups, respectively, (v) no significant difference in the staining scores of all antibodies between the IUGR and preeclampsia groups.

Discussion

Preeclampsia is a systemic disease that is characterized by diffuse endothelial dysfunction, vasospasm, increased oxidative and/or nitrosative stress and activation of the coagulation system. Previous studies have shown that placentas from pregnancies complicated by preeclampsia or IUGR show enhanced apoptosis as compared to placentas from normal pregnancies (Smith et al. 1997b; DiFederico 1999; Allaire et al. 2000). The role of apoptosis has also been demonstrated in trophoblasts under hypoxic conditions (Levy et al. 2000). Moreover, a relationship between enhanced p53 expression and trophoblast apoptosis was suggested in pregnancies complicated by fetal growth restriction (Levy et al. 2002). Indeed, the mechanisms that lead to placental damage are very complex. In a balance with p53, Ishihara et al. (2000) have shown increased apoptosis throughout fas antigen and bcl-2 in syncytiotrophoblast in human term placentas complicated by either preeclampsia or IUGR. In concordance with previous findings, we have observed increased human placental apoptosis as shown by caspase-3 staining in both IUGR and preeclampsia. Hence, our study indicates caspase related apoptosis, as judged by the M30 staining which is a specific marker for epithelial apoptosis (Kadyrov et al. 2001).

Numerous stimuli, including oxidative stress, tumor necrosis factor-α, and high glucose are known to induce apoptosis in endothelial and epithelial cells (Muschell et al. 1995; Haimovitz-Friedman 1997). These stimuli also increase NF-κB binding activity which has an important role in cell-death pathways in endothelial cells (Kitajima et al. 1996; Morigi et al. 1998). Tumor necrosis factor-α can cause programmed cell death and this is often preceded by increased NF-κB activation (Beyaert and Fiers 1994). Activation of NF-κB binding activity has been observed in endothelial cells under hypoxic conditions and numerous studies revealed that the activation of NF-κB induced by hypoxia causes endothelial cell death through bcl-2 dependent apoptosis. Bcl-2 also causes a decrease in NF-κB activity (Badriachini et al. 1996; Grimm et al. 1996). In the present study, we have found increased NF-κB expression in placental trophoblastic epithelium from IUGR and preeclampsia. Increased NF-κB as observed in this study may have resulted from decreased bcl-2 expression. It has been shown that one of the functions of p53 is to repress expression of bcl-2 gene (Wu et al. 1994) and we can only speculate that the down-regulation of bcl-2 may cause activation of NF-κB, which may be mediated by the induction of p53. Additionally, bcl-2 overexpressing cells have elevated pools of antioxidants and NF-κB activation is regulated by the intracelluler redox status (Voehringer 1999; Jang and Surh 2003). There appears to be a clear link between bcl-2 and NF-κB which also supports our conclusion that the balance between bcl-2 and NF-κB is altered in plancentas of IUGR and preeclamptic patients.

On the other hand, there is also increasing evidence on the role of NF-κB in the regulation of antiapoptotic gene expression. The multiface of NF-κB is believed to inhibit apoptosis in caspase dependent pathways, and numerous reports documented antiapoptotic action of NF-κB (Kitajima et al. 1996; Qin et al. 1998). Hence, the exact mecanism of NF-κB effect on the apoptotic pathways is still controversial.

The correlation of lipid peroxides and NF-κB have also been shown in vascular endothelial cells (Takacs et al. 2001). Antioxidants such as vitamin E and N-acetylcysteine have been shown to effectively prevent NF-κB activation and the upregulation of ICAM-1 expression in human umbilical cells that have been processed from plasma of preeclamptic women. As the potential role of apoptosis in the pathophysiology of pre-

eclampsia and IUGR has been documented, NF-κB levels might be modulated by effective antioxidant therapies. However, little is known about the signaling pathway so far, and apart from the present work, to the best of our knowledge there has been no other study that investigates the relationship between the increased expression of NF-κB and the increased apoptotic changes in trophoblastic cells of IUGR and preeclampsia.

In the present study, the increased placental trophoblastic apoptosis as shown by M30 and caspase-3 staining was accompanied by increased NF-κB and decreased bcl-2 expression in pregnancies complicated with IUGR and pre-eclampsia. Based on these observations, we speculate that placental trophoblastic apoptosis might have resulted from NF-κB dependent pathway. Furthermore, increased staining with NF-κB in these groups have led us to conjecture that this overexpression might be due to interaction of bcl-2. Still, it is not clear whether NF-κB forced diminished bcl-2 expression or reduced bcl-2 expression forced the activation of NF-κB. To this end, future research is necessary to fully understand the relationship between bcl-2 and NF-κB in apoptotic cascade.

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