A Comparative Study of Apoptosis in Placentas of Normal and Preeclamptic Indian Pregnant Women by TUNEL Assay and M30 Immunostaining

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Background: Preeclampsia is a unique life-threatening disorder of human pregnancy associated with the abnormal placentation caused by the inadequate trophoblastic invasion due to altered apoptosis of these cells. The aim of the present study was to assess and compare the apoptosis in trophoblastic cells in various zones (villous and extravillous) of placentas of preeclamptic and normotensive nonproteinuric pregnant women. **Methods:** Hematoxylin eosin staining, TUNEL assay and M30 immunostaining techniques were used for studying apoptosis in trophoblastic cells of placentas of two groups. The results of apoptotic indices by these techniques were compared in preeclamptic group. **Results:** The TUNEL apoptotic indices were higher in all the

zones of placentas of preeclamptic group as compared to control group though the results were not statistically significant. M30 immunostaining also gave higher apoptotic indices in all the zones of preeclamptic placentas as compared to the normal group but the result of apoptotic index of basal plate was not statistically significant. M30 immunostaining was absent in stromal cells.

Conclusion: In our study, the trophoblastic apoptotic rates were more in villous as well as extravillous zones of preeclamptic placentas as compared to normal placentas and M30 immunostaining was found to be more sensitive and specific for the apoptotic trophoblastic cells as compared to TUNEL assay. *J. Clin. Lab. Anal.* 26:459–466, 2012. © 2012 Wiley Periodicals, Inc.

Key words: apoptosis; DNA fragmentation; immunohistochemistry

INTRODUCTION

Preeclampsia, a syndrome observed during second half of pregnancy, thought to be caused by a combination of immunological, environmental, nutritional, and genetic factors, has been described as “disease of theories.” It is defined as the onset of persistent hypertension exceeding 140/90 mmHg associated with proteinuria exceeding 300 mg/day after 20th week of gestation in previously normotensive nonproteinuric pregnant women (1). The clinical manifestations of preeclampsia are associated with capillary leak, hypercoagulation due to the diffuse endothelial damage (2).

The prerequisite for the development of preeclampsia is the abnormal placentation leading to placental ischemia and hypoxia (3). The “trophoblastic cell” is considered to be the main culprit of this disease of pregnancy. The trophoblastic cell undergoes apoptosis during normal placental development. As pregnancy progresses, the placental apoptosis increases suggesting that it could play a role

in the normal development and aging of placenta (4). Various efforts have been done to identify apoptotic cells of placentas and these include light and electron microscopy (5), DNA fragmentation analysis by electrophoresis (6), in situ nick end labeling (7), TUNEL assay (8), annexin v detection (9), and M30 immunostaining (10). M30 antibody detects cytoplasmic cytokeratin cleavage products produced during apoptotic cascade (11) whereas TUNEL assay identifies endonuclease-mediated DNA breakdown in the nuclei of apoptotic cells. A combination of two or more assays may be more appropriate to detect the majority of cells undergoing apoptosis at one time. Thus, in

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the present study, we have made an attempt to localize the apoptotic trophoblastic cells in various zones of normal and preeclamptic placentas using both TUNEL assay and M30 immunostaining so that we can identify the cells in various stages of apoptosis. The appropriate assessment of apoptotic activity may find its clinical relevance in predicting the progression of the disease not only in case of preeclampsia but also other heterogeneous set of diseases arising from abnormal trophoblastic tissue.

MATERIALS AND METHODS

Sample Collection

A total of 40 placentas were collected from the labor room of which 20 were from normotensive nonproteinuric pregnant women and 20 were from preeclamptic pregnant women with gestational ages of 30–42 weeks. The protocol of the study was approved by the ethical committee and consent was obtained from each individual for the study group. Preeclampsia was diagnosed in nulliparous women with no history of hypertension prior to pregnancy, increase in diastolic pressure of 15 mmHg or systolic pressure of 30 mmHg compared with blood pressure obtained before 20th week of gestation, and proteinuria >30 mg/dl. Patients with chorioamnionitis, chronic hypertension, pregestational diabetes, renal disease, cardiac disease, active asthma, thyroid disease and pre-existing seizure disorder, and HELLP syndrome were excluded from this study.

The samples were taken from the central part of the placental disc and areas of visible infarcts, calcification, or hematomas were excluded. The samples were then fixed in 10% formaldehyde and processed for hematoxylin and eosin staining, TUNEL assay, and M30 immunostaining (Roche Diagnostic, Germany). All the techniques were standardized according to ambient conditions of our laboratory.

In TUNEL assay, tissue sections were deparaffinized and rehydrated according to standard protocols. The slides were permeabilized by using 0.1% sodium citrate and quenching was done by 3% H_2O_2 in methanol. TUNEL reaction mixture was prepared by adding 50 μl of enzyme solution (terminal deoxynucleotidyl transferase enzyme isolated from calf serum) to 450 μl of label solution (Biotinylated nucleotide mixture in reaction buffer) and incubated for 60 min at 37°C in a humidified chamber. Then, the samples were incubated in 50–100 μl of peroxidase substrate containing streptavidin conjugated to horseradish peroxidase for 10 min at 15–25°C. After each step, the slides were washed with phosphate buffered saline. The slides were counterstained in hematoxylin and finally mounted with permanent mounting media (DPX). An adjacent serial section pretreated with

DNase I was used as a positive control. The tissue sections not treated with Tdt enzyme were used as negative control. All 40 samples of placenta were analyzed for expression of apoptotic nuclei and digital images of ten randomly selected high-power fields were obtained using a Nikon microscope (Nikon Corp, Tokyo, Japan). The sections were examined by two independent observers. Approximately 1000–3000 nuclei were counted for each placenta in preeclamptic and control groups. Apoptotic nuclei were easily differentiated from nonapoptotic by their brown labeling and condensed heterochromatic nucleus. The total number of nuclei (apoptotic nuclei + live nuclei) and total number of apoptotic nuclei were counted. The TUNEL apoptotic index (Total number of apoptotic nuclei per total number of nuclei multiply by 100) was calculated in various zones of placenta, e.g., in villous zone (trophoblast cells, syncytial knots, stromal cells) and in extravillous zone (trophoblastic cells in chorionic plate and basal plate).

In M30 immunostaining, tissue sections were prepared as for the TUNEL assay. All subsequent steps were carried out at room temperature in a humidified chamber. The peroxidase activity was blocked by using 3% H_2O_2 prepared in methanol for 10 min. The specimens were then incubated in 1–3 drops of serum block for 2 hr to prevent nonspecific binding to collagen and connective tissue. The specimens were incubated for 24 hr with M30 antibody (1:50) followed by 1–3 drops of biotinylated secondary antibody (antimouse IgG) for 30 min. The specimens were thereafter incubated in 1–3 drops of HRP-streptavidin complex for 30 minutes. The slides were then counterstained with hematoxylin and dehydrated by passing through ascending grades of ethanol and slides were mounted with DPX and were observed under light microscope. The negative tissue control included eliminating the primary antiserum and replacing species-specific antiserum with normal horse serum. Sections of colon adenocarcinoma served as positive control. All 40 samples of placenta were analyzed for expression of cytokeratin neopeptide in the apoptotic cells using M30 antibody staining in serially sectioned slides. Cells with the brown cytoplasmic stain were considered M30 immunostaining positive. M30 apoptotic index was calculated in villous zone (trophoblast cells, syncytial knots, stromal cells) as well as in extravillous zone (trophoblastic cells in chorionic plate and basal plate) of normal and preeclamptic placentas.

The clinical characteristics (blood pressure, urinary protein, maternal age, gestational age) were compared among the two groups.

Various histological features were studied in both the normal and preeclamptic placentas using hematoxylin and eosin staining. The comparison between apoptotic indices in various zones of normal and preeclamptic

placentas was done by using both the techniques. The results of apoptotic indices were also compared among the techniques in preeclamptic placentas.

Statistical Analysis

Statistical analysis was carried out using Stata 9.0/Data analysis software (college Station, Texas). Data were presented as number (%) or mean $\pm$ SD/Median (range) as appropriate. Quantitative variables between the groups were compared using student "t" test for independent samples/Wilcoxon rank sum test. Categorical variables were compared between the groups using Chi square test. The *P* value less than 0.05 was considered statistically significant.

RESULTS

Clinical Characteristics of Preeclamptic and Control Group (Tables 1 and 2)

The mean systolic and diastolic pressures in preeclamptic group were 142.5 ± 8.7 and 96.3 ± 9.5 mmHg, respectively whereas in control group, the systolic and diastolic pressures were 112 ± 8.6 and 79.7 ± 6.1 mmHg, respectively. There was a significant difference in the systolic and diastolic pressures in both the groups ($P < 0.0001$). Urinary protein was determined in 24-hr urine samples of preeclamptic and control groups. The level of protein in the preeclamptic group was 1.85 (0.5 – 3.9) g/day as compared to 0.16 (0.1 – 0.29) g/day in the control group. There was a significant difference in urinary protein levels in preeclamptic and control groups ($P < 0.0001$). Thus, there was increase in blood pressure and protein levels in preeclamptic patients as compared to control group.

TABLE 1. Clinical Characteristics of Preeclamptic and Control Groups

Clinical characteristics	Mean $\pm$ SD (range)		Difference in means 95% CI	<i>P</i> value
	Preeclampsia (<i>n</i> = 20)	Control (<i>n</i> = 20)		
Blood pressure				
Systolic (mmHg)	142.5 ± 8.7 (120–160)	112 ± 8.6 (100–124)	11.09 (107.9–138.3)	0.0001
Diastolic (mmHg)	96.3 ± 9.5 (82–118)	79.7 ± 6.1 (72–90)	6.54 (76.8–91.8)	0.0001

n = number of subjects.

Data are presented in Mean $\pm$ SD (range).

Student *t*-test for independent samples.

CI, confidence interval. $P < 0.05$, statistically significant.

TABLE 2. Clinical Characteristics of Preeclamptic and Control Groups

Clinical characteristics	Median (range)		<i>P</i> value
	Preeclampsia (<i>n</i> = 20)	Control (<i>n</i> = 20)	
Urine Protein (gm/day)	1.85 (0.5 – 3.9)	0.16 (0.1 – 0.29)	0.0001

n = number of subjects.

Data is presented in Median (range).

Two samples Wilcoxon rank sum (Mann Whitney Test).

$P < 0.05$, statistically significant.

TABLE 3. Obstetric Characteristics of Preeclamptic and Control Groups

Obstetric characteristics	Mean $\pm$ SD (range)		Difference in means 95% CI	<i>P</i> value
	Preeclampsia (<i>n</i> = 20)	Control (<i>n</i> = 20)		
Maternal age (years)	31.25 ± 4.2 (25–38)	27.65 ± 3.6 (20–34)	2.88 (25.9–29.2)	0.0064
Gestational age (weeks)	35.1 ± 2.7 (31–40)	38.25 ± 1.4 (36–41)	4.55 (37.5–33.8)	0.0001

n = number of subjects.

Data are presented in Mean $\pm$ SD (range).

Student *t*-test for independent samples.

CI, confidence interval.

$P < 0.05$, statistically significant.

Obstetric Characteristics of Preeclamptic and Control Group (Table 3)

The average maternal age was 31.25 ± 4.2 years in preeclamptic group as compared to 27.65 ± 3.6 years in control group and their gestational age was 35.1 ± 2.7 and 38.25 ± 1.4 weeks, respectively. Thus, the maternal age was higher in preeclamptic groups as compared to the control (*p* value 0.0064). However, the gestational age was low in preeclamptic group as compared to control ($P < 0.0001$).

Comparison of Obstetric Characteristics of Preeclamptic and Control Group (Table 4)

The maternal age was more than 30 years in 50% of preeclamptic and 25% of control group. The gestational age was less than 37 weeks in 80% of preeclamptic and 10% of control groups. Thus, the higher percentage of preeclamptic group had increased maternal age and reduced gestational period.

The placentas from preeclamptic patients showed areas of hematomas, infarcts, calcification, and fibrosis. On histological examination, they showed slightly disturbed pattern of villi. These villi were of variable sizes like small,

medium, and large. The villi consisted of core of mesenchyme, large number of blood vessels, and were lined by cytotrophoblast and syncytiotrophoblast. The small-sized terminal villi were less and distributed wider apart. There was increased deposition of fibrin plaques on the trophoblastic surface. There was increased trophoblastic proliferation and increased villous capillary growth that caused deformation of the villi. Increased number of syncytial knots were observed in preeclamptic placentas (Fig. 1).

The TUNEL assay gave higher apoptotic indices in all the zones of preeclamptic placentas as compared to normal placentas though the results were not statistically significant (Fig. 1, Table 5).

The M30 immunostaining also gave higher apoptotic indices in all the zones of preeclamptic placentas as compared to normal placentas but the result of apoptotic index in the trophoblastic cell of basal plate was not statistically significant among the two groups. M30 immunostaining was absent in the villous stromal cells of

Fig. 1. Photomicrographs of villous part of placenta. (A) Normal placenta showing syncytiotrophoblast (single arrow), cytotrophoblast (double arrow) and stroma (star). (B) Preeclamptic placenta showing altered villous morphology (arrow) and RBCs in intervillous space (star). (C) Normal placenta showing TUNEL assay positive (brown stained) nuclei in the villous stroma (arrow). (D) Preeclamptic placenta showing TUNEL assay positive (brown stained) syncytial knot (arrow). (E&F) Preeclamptic placenta showing M30 immunostaining positive (brown stained) syncytial knots (arrows). Scale bar 100µm.

TABLE 4. Comparison of Obstetric Characteristics in Preeclamptic and Control Groups

Obstetric characteristics	Number of subjects (%)		OR (95% CI)	P value
	Preeclampsia (n = 20)	Control (n = 20)		
Maternal age (years)				
≤30	10 (50)	15 (75)	2.7 (0.77–10.04)	0.11
>30	10 (50)	5 (25)		
Gestational age (weeks)				
≤37	16 (80)	2 (10)	16.71 (2.97–93.88)	0.001
>37	4 (20)	18 (90)		

n = number of subjects, Chi square or Fisher's exact test was applied.

OR, odds ratio; CI, confidence interval.

Percentage (%) indicated in parenthesis. $P < 0.05$, statistically significant.

TABLE 5. Percentage of Apoptotic nuclei (Apoptotic Index) With TUNEL Assay in Preeclamptic and Control Groups

Different sites used for counting of nuclei	Apoptotic index (Mean ± SD [range])		P value
	Preeclampsia (n = 20)	Control (n = 20)	
Villous zone			
1. Trophoblastic cells	58.2 ± 12.3 (37.5–87.5)	36.9 ± 10.1 (11.1–58.3)	0.151
2. Syncytial knots	89.3 ± 13.1 (25–60)	74.2 ± 28.5 (0–37.5)	0.164
3. Stromal cells	27.8 ± 12.3 (10–50)	26 ± 12.4 (16.6–42.6)	0.647
Extravillous zone			
1. Chorionic plate	23 ± 8.7 (14.2–42.8)	13.9 ± 5.5 (7.1–16.6)	0.250
2. Basal plate	33.8 ± 14.5 (16.6–57.1)	42.7 ± 12.7 (9–27.2)	0.465

n = number of subjects.

Data is presented in mean ± SD (range).

Student t test for independent sample.

$p < 0.05$, statistically significant.

TABLE 6. Percentage of Apoptotic Nuclei (Apoptotic Index) With M30 Immunostaining in preeclamptic and control groups

Different sites used for counting of M30 positive cells	Apoptotic index (Mean ± SD [range])		P value
	Preeclampsia (n = 20)	Control (n = 20)	
Villous zone			
1. Trophoblastic cells	92 ± 6.3 (76.3–99)	61.9 ± 14.4 (37.3–87.9)	0.0001
2. Syncytial knots	73.6 ± 20.5 (25–100)	36.9 ± 20 (0–66.6)	0.0001
3. Stromal cells	0	0	0
Extravillous zone			
1. Chorionic plate	37.4 ± 15.1 (13.8–75)	24.5 ± 8.6 (8.8–37.5)	0.002
2. Basal plate	37.1 ± 12 (18.5–62.5)	30.5 ± 13.1 (9–60)	0.1

n = number of subjects.

Data are presented in mean ± SD (range).

Student *t*-test for independent sample.

$P < 0.05$, statistically significant.

in the villous stromal cells whereas M30 immunostaining was not seen in these cells (Fig. 2, Table 7).

DISCUSSION

The Preeclampsia has been associated with increased maternal age (12). In our study also, the maternal age in preeclamptic group was significantly higher as compared to control group. The pathophysiology of preeclampsia involves the dysfunctioning of trophoblastic cells leading to poor implantation and failure of spiral artery remodeling causing reduced placental perfusion, ultimately leading to intrauterine growth restriction and premature delivery (13). The results of our study show that preeclampsia is associated with decreased gestational age. The pathogenesis of preeclampsia could be due to altered apoptotic rate of trophoblastic cells (14). Our study results show that the apoptotic indices of TUNEL assay as well as M30 immunostaining were higher in preeclamptic placentas as compared to normal placentas. This finding suggests that the excessive apoptosis of trophoblastic cells leads to failure of placental invasion and hence implantation. Thus, a clear and better understanding of placental apoptosis and its regulation is important as it can provide newer insights into placental pathologies potentially suggesting therapeutic targets. This in turn necessitates the need to find methods that accurately identify the apoptotic cells. One of the changes occurring in the cells undergoing apoptosis is the DNA fragmentation by endonucleases. This change occurs very late during apoptosis cascade for a very brief period of time (15). Moreover, this DNA fragmentation

both normal as well as preeclamptic placentas (Fig. 1, Table 6).

On comparing the results of these two techniques in preeclamptic placentas, the M30 immunostaining gave higher apoptotic indices in villous zone trophoblastic cells and extravillous trophoblastic cells of chorionic plate and basal plate cells as compared to TUNEL assay though the result of apoptotic index of the trophoblastic cells of basal plate was not statistically significant. TUNEL assay gave a higher apoptotic index of the syncytial knots as compared to M30 immunostaining but this result was not statistically significant. TUNEL assay showed positive staining

Fig. 2. Photomicrographs of comparison between TUNEL assay and M30 immunostaining. (A) Preeclamptic placenta showing TUNEL assay positive (brown stained) nuclei in the villous stroma (arrow). (B) Preeclamptic placenta showing M30 immunostaining positive cytoplasm (brown stained) in the villous zone (arrow). (C) Preeclamptic placenta showing TUNEL assay positive (brown stained) nuclei in the basal plate (arrow). (D) Preeclamptic placenta showing M30 immunostaining positive (brown stained) cytoplasm in the basal plate (arrow). Scale bar 100µm.

TABLE 7. Comparison Between M30 Immunostaining and TUNEL Assay in Preeclamptic Placenta

Different sites used for counting	Apoptotic index (Mean $\pm$ SD [range])		<i>P</i> value
	M30 (<i>n</i> = 20)	TUNEL (<i>n</i> = 20)	
Villous zone			
1. Trophoblastic cells	92 $\pm$ 6.3 (76.3–99)	58.2 $\pm$ 12.3 (37.5–87.5)	0.004
2. Syncytial knots	73.6 $\pm$ 20.5 (25–100)	89.3 $\pm$ 13.1 (25–60)	0.25
3. Stromal cells	0	27.8 $\pm$ 12.3 (10–50)	0.001
Extravillous zone			
1. Chorionic Plate	37.4 $\pm$ 15.1 (13.8–75)	23 $\pm$ 8.7 (14.2–42.8)	0.047
2. Basal plate	37.1 $\pm$ 12 (18.5–62.5)	33.8 $\pm$ 14.5 (16.6–57.1)	0.55

n = number of subjects.

Data are presented in mean $\pm$ SD (range).

Student *t*-test for independent sample.

P < 0.05, statistically significant.

also occur in the necrotic cells (16). Therefore, TUNEL assay may not be a sensitive and specific test for identifying only the apoptotic cells. Other characteristic feature of the epithelial cell apoptosis is the caspase-mediated cleavage of cytokeratins leading to disorganization of the cytoskeleton. Proteolysis of cytokeratins 18, 19 is involved in apoptosis degradation. These apoptosis-specific proteins have been identified as markers of increased cellular turnover (17, 18). Cytokeratin 18 has widespread distribution and is largely expressed in simple, nonstratified, ductal, and pseudostratified epithelia along with normal trophoblasts. Cytokeratin 18 when cleaved by caspase 3, 7 liberates a neoepitope, which can be identified by the M30 monoclonal antibody even before the disruption of membrane asymmetry and DNA strand break occurs (19). Thus, M30 immunostaining becomes an important tool for identifying apoptosis at very early stages.

In our study, the M30 immunostaining did not give a significant difference between the apoptotic index of basal plate trophoblastic cells of the two groups. This can be due to different amounts of basal plate tissue taken as placentas were collected after the normal separation

of placenta and not by manual curettage. Therefore, the amount of basal plate tissue that we got, might be different for each placenta.

The comparison between the two techniques shows that M30 immunostaining is more sensitive and specific in identifying the apoptotic trophoblastic cells. This is due to the fact that M30 immunostaining becomes positive at an early stage of apoptosis and it remains positive for a longer time but when cells become necrosed, its positivity is lost. The higher TUNEL apoptotic index for stromal cells as compared to M30 apoptotic index indicates that M30 immunostaining is specific for cells of epithelial origin like trophoblastic cells whereas TUNEL assay identifies all apoptotic cells of various lineages.

In preeclamptic placentas, the syncytial knots of villous zone showed a higher TUNEL apoptotic index as compared to the M30 apoptotic index, though the difference was not statistically significant. In preeclampsia, the excessive rate of trophoblastic apoptosis may cause the increased syncytial knot formation (20), which was clearly shown with our results. As the apoptotic nuclei of these syncytial knots accumulate, the M30 antibody recognizes the M30 neoepitope formed as a result of cytokeratin breakdown and ultimately cytoplasmic staining is seen. When these syncytial knots get detached from the villous surface toward the end of the apoptotic cascade, the TUNEL assay identifies the fragmented DNA and nuclear staining is seen. In preeclamptic placentas, these syncytial knots may undergo necrosis and TUNEL assay identifies these necrosed syncytial knots.

During our study, we could demonstrate three subsets of cells based on the their staining pattern:

- (1) Cells positive for both M30 immunostaining and TUNEL assay. These cells are likely to be trophoblastic cells in very final stages of apoptosis.
- (2) Cells positive for M30 immunostaining but not for TUNEL assay. These cells are at the execution stage of apoptosis, i.e., after cytokeratin cleavage, prior to activation of endonucleases and degradation of DNA.
- (3) Cells positive for TUNEL assay but not for M30 immunostaining. These cells are nontrophoblastic cells or cells in late stages of apoptosis or cells undergoing necrosis.

With the results of our study, we confirm that one of the pathogenetic mechanisms of preeclampsia is the exaggerated apoptosis of trophoblastic cells, so the search for the most sensitive marker becomes the utmost priority. Moreover, our study also suggest that monoclonal antibody M30 may be a useful tool for studying trophoblast apoptosis. The antibody is easy to handle, and the CK18 neoepitope recognized seems specific for apoptosis. This appears to be the first study of apoptotic markers and

apoptotic index in placentas of preeclamptic patients in India where trophoblastic cells not only from villous but from various zones of placenta have been studied, which makes our study more elaborative and authentic. Therefore, our study can be used as baseline for future prospective study on larger cohorts. Moreover, further studies are required to determine the regulating factors of apoptosis of trophoblastic cells, which may provide newer insights into not only the understanding of molecular basis of pathophysiology of preeclampsia but also in identifying various other assays and immunostainings for localizing the apoptotic cells in placenta and various other tissues.

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