Differential apoptotic activity in trophoblast of spontaneous abortions and normal pregnancies

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Abstract

Introduction. Apoptosis is a key process during normal trophoblastic development and, consequently, the whole gestation. However, in trophoblastic differentiation in spontaneous abortions apoptosis has been hardly investigated. Therefore, the aim of the study was to investigate the correlation between apoptotic frequency in trophoblast and spontaneous abortion incidences.

Material and methods. A total of 72 trophoblastic tissue samples were immunohistochemically examined. 42 of 72 derived from first-trimester spontaneous abortions and the remaining 30 from elective terminations during the same trimester of pregnancy. TUNEL assay and M30 marker were used for apoptosis evaluation by immunohistochemistry.

Results. Comparative study of tissues from spontaneous abortions and elective pregnancy terminations demonstrated increased expression of both apoptotic markers in tissues derived from spontaneous abortions compared to normal pregnancies. In addition, statistical analysis correlated maternal age and gravidity with increased spontaneous abortion incidences. Moreover, both M30 and TUNEL staining were significantly correlated with maternal age and primigravidity in spontaneous abortion cases.

Conclusions. Our data proved that elevated apoptotic activity during the first pregnancy trimester is clearly involved in spontaneous abortions. Moreover, two well-established apoptotic markers revealed high statistical significance in the evaluation of post-abortive tissues. (Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 1, 24–30)

Key words: spontaneous abortion; trophoblast; apoptosis; TUNEL assay; M30 marker; IHC
Introduction

Spontaneous abortion referred to as a self-generating loss of a fetus before the 20th week of gestation represents a multifactorial and common complication of early pregnancy and affects about 15% of clinically diagnosed pregnant women [1, 2]. Chromosomal fetal aberrations, uterine anatomic defects, sperm pathology, and environmental causes are some of the possible risk factors [3, 4].

Despite the progress in understanding the mechanisms of implantation and placentation, the pathophysiology of spontaneous abortions remains unclear. It is well known that apoptosis, either intrinsically or extrinsically activated, is a fundamental process involved during embryogenesis [5]. Pregnancy maintenance requires a gold balance between proliferation and apoptosis of trophoblast cells [6]. More specifically, programmed cell death mediated by the FasR/FasL system is necessary for blastocyst implantation, as extreme apoptotic activity of endometrial epithelium is observed after blastocyst’s attachment [7]. Apoptosis is also involved in maternal immune tolerance during trophoblast invasion [8]. Furthermore, cytотrophoblast differentiation and syncytial fusion seem to be triggered by apoptotic activity via Caspace 8 [9]. On the other hand, increased placental apoptotic activity is related to preeclampsia by blocking trophoblast invasion into spiral arteries [10].

In this study, we compared the apoptotic rate in trophoblast of spontaneous abortions and elective terminations, using M30 immunostaining and TUNEL assay. Moreover, we determined the statistical correlation of maternal age and gravidity (number of pregnancies a woman has had) with spontaneous abortions and associated the maternal age and gravidity with M30 expression and TUNEL staining. The diagnostic accuracy of both apoptotic markers in trophoblast coming from spontaneous abortions was also assessed.

Material and methods

Tissue specimens. Seventy-two trophoblast samples were obtained from the Laboratory of Histology-Embryology archive, Medical School, Democritus University of Thrace, Greece. 42 of 72 resulted from first-trimester spontaneous abortions (SA) and 30 from elective terminations (ET), which were regarded as controls. All SA specimens had been excluded for genetic or anatomic abnormalities, infections, maternal immune, hematological or endocrine diseases, while all ET specimens were derived from healthy women. Clinical data could be retrieved in all cases.

Ethical considerations. Ethical approval was obtained from the Scientific Committee and the Ethics Research Committee of the University Hospital of Alexandroupolis, Alexandroupolis, Greece (No 417). The study was conducted according to the criteria set by the 1964 Helsinki Declaration and its later amendments.

Immunohistochemistry and TUNEL assay. The samples were fixed in 10% buffered formalin and embedded in paraffin according to the standard histological protocol. Histopathologic examination was performed at 4-μm hematoxylin-eosin-stained sections. Serial sections from each case were deparaffinized, rehydrated, and treated with 0.3% H2O2 for 15 min at room temperature (RT) to prevent endogenous peroxidase activity. The immunohistochemical staining was imaged by the Dako detection system (Real EnVision, Detection System kit, Dako, Glostrup, Denmark). This technique is based on the development of a complex between avidin-biotin antibody and the tissue’ antigenic epitopes. The Dako EnVision chromogen (AEG, Dakocytomation) was used to visualize the aforementioned complex.

M30 CytoDeath (Roche Diagnostics, Mannheim, Germany) kit and TUNEL assay (PROMEGA, Madison, WI, USA) were used in this study as immunohistochemical markers of apoptosis. M30 is a widely used early apoptotic marker resulting from a caspase cleavage site within cytokeratin 18, a cytoskeleton component of the trophoblasts [11], while TUNEL staining detects cells in the final steps of the apoptotic cascade using the terminal transferase activity (TdT) [12]. For M30 staining, slides were incubated for 60 min with monoclonal antibody (No 12140349001) at 1:100 dilution. TUNEL staining occurred according to the manufacturer’s instructions. All samples were examined using a Nikon Eclipse 50i microscope with an integrated camera Nikon Digital Sight DS-L1 (Nikon Corporation, Tokyo, Japan).

Immunohistochemical score of M30 protein expression in trophoblast. From each slide, 5 high-power fields were randomly chosen. The positive expression of M30 was determined by counting the number of stained cells. Each slide was evaluated and scored by a semi-quantitative scoring system in which both staining intensity [Staining Intensity (SI)] and the extent of it [Positive Percentage (PP)] were taken into account. Thus, for each histological section, the obtained score was based on both the intensity (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) and the extent of the stained cells (0% = 0, 1–10% = 1, 11–50% = 2, 51–80% = 3, 81–100% = 4). The final score (PPSI) emerged as the result of Intensity × Extent, with a minimum score of 0 and a maximum score of 12 [13].

TUNEL assay evaluation. To avoid overestimation of apoptosis, the evaluation of TUNEL assay was based on positive TUNEL staining for fragmented DNA as well as on both...
morphological features of apoptosis (nuclear condensation, cytoplasmic fragmentation). For each slide, positive cells were counted in 3 non-overlapping higher-power fields in the same position. Apoptotic index (AI), defined as the number of TUNEL positive cells/total count of nuclei ×100% was estimated.

Statistical analysis. Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS), version 19.0 (IBM Corp., Armonk, NY, USA). M30 (PPSI) and TUNEL (AI) values were considered as quantitative variables and they were expressed as mean ± standard deviation SD; median values and range were also presented. The normality of the other quantitative variables (maternal age, gravidity) was tested with Kolmogorov-Smirnov test and they were expressed as mean ± standard deviation (SD). Qualitative variables were expressed as absolute and relative (%) frequencies.

To assess differences in clinical characteristics between the two groups, Student’s t-test (age), Mann-Whitney U test (M30, TUNEL assay), and chi-square test (gravidity) were used. One-way analysis of covariance (ANCOVA) was also performed to correlate the differences of M30 (PPSI) and TUNEL assay (AI) levels between the two groups with maternal age and gravidity. Spearman’s ρ correlation coefficient was used to evaluate any potential association of M30 (PPSI) and TUNEL (AI) levels with patients’ age.

For the evaluation of the diagnostic accuracy of M30 (PPSI) and TUNEL (AI), the area under the receiver operating characteristic (ROC) curve (AUC) was calculated. Sensitivity, specificity, positive and negative predictive values were also calculated, while Cohen’s kappa was used to assess agreement. The value with the shortest distance from the curve to the point with both maximum sensitivity and specificity, i.e., the point (0.0, 1.0), was selected as the cut-off score to classify patients with spontaneous abortions. All tests were two-tailed and statistical significance was considered for p values < 0.05.

Results

Association of spontaneous abortions’ rates with maternal age and gravidity

At first, we compared the maternal age of women with spontaneous abortions (SA) with women proceeding to elective terminations (ET). A statistically significant difference in maternal age between the two groups was observed, with the age of women with spontaneous abortions being higher (31.29 ± 6.42 vs. 28.23 ± 5.90 in SA and ET, respectively, p = 0.040) (Table 1). Similarly, statistically significant differences were noted with regards to primigravidity and the occurrence of spontaneous abortions (p < 0.001). More specifically, the percentage of spontaneous abortions was higher in primigravida (pregnant for the first time) women than in the control group (64.2% vs. 36.7%, p < 0.001), while multigravida women (women has been pregnant more than two times) had higher frequencies in the control group compared to those with spontaneous abortions (40% vs. 2.8%, p < 0.001) (Table 1).

Differential apoptotic activity between elective pregnancy terminations and spontaneous abortions

The expression levels of the M30 marker and TUNEL were measured in both SA and ET cases (Table 1). Enhanced apoptotic activity was clearly noted in the spontaneous abortions group, as compared to the control group (Table 1). In trophoblasts of the SA group, M30 was expressed both in the cytoplasm and cell nucleus. It was predominantly detected in syncytiotrophoblast, however, cytotrophoblastic expression was also present (Fig. 1 A1). TUNEL detection in

Table 1. Comparison of maternal age, gravidity, and two apoptotic markers, M30 immunopositivity, and TUNEL staining, between spontaneous abortions and elective terminations groups

<table>
<thead>
<tr>
<th></th>
<th>Elective terminations</th>
<th>Spontaneous abortions</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years, mean ± SD)</td>
<td>28.23 ± 6.42</td>
<td>31.29 ± 5.90</td>
<td>0.040</td>
</tr>
<tr>
<td>Gravidity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (first)</td>
<td>11 (36.7%)</td>
<td>27 (64.2%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>II (second)</td>
<td>7 (23.3%)</td>
<td>13 (31.0%)</td>
<td></td>
</tr>
<tr>
<td>&gt; II</td>
<td>12 (40.0%)</td>
<td>2 (2.8%)</td>
<td></td>
</tr>
<tr>
<td>M30 expression (PPSI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.70 ± 0.47</td>
<td>4.33 ± 1.30</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Median value (min–max)</td>
<td>1.00 (0.00–1.00)</td>
<td>4.00 (1.00–6.00)</td>
<td></td>
</tr>
<tr>
<td>TUNEL assay (AI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.77 ± 0.43</td>
<td>4.05 ± 1.55</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Median value (min–max)</td>
<td>1.00 (0.00–1.00)</td>
<td>4.00 (1.00–6.00)</td>
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</tbody>
</table>

AI — apoptotic index; PPSI, a score received by multiplying intensity and extent of immunohistochemical reaction (see Methods).
Trophoblast apoptosis in spontaneous abortions

**Table 2.** Correlation of M30 (PPSI) expression or TUNEL (AI) staining with maternal age and gravidity in spontaneous abortions and elective terminations groups

<table>
<thead>
<tr>
<th></th>
<th>M30 (PPSI)</th>
<th>P value</th>
<th>TUNEL (AI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Elective terminations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Age (Spearman’s p)</td>
<td>0.266</td>
<td>0.156</td>
<td>–0.005</td>
<td>0.981</td>
</tr>
</tbody>
</table>
| Gravidity
  I                | 0.73 ± 0.47| 0.812   | 0.73 ± 0.47| 0.710   |
  ≥ II            | 0.68 ± 0.48|          | 0.79 ± 0.42|         |
| **Spontaneous abortions**|            |         |            |         |
| Maternal Age (Spearman’s p) | 0.458      | 0.002   | 0.459      | 0.002   |
| Gravidity
  I                | 4.63 ± 1.33| 0.036   | 4.41 ± 1.67| 0.022   |
  ≥ II            | 3.80 ± 1.08|          | 3.40 ± 1.06|         |

AI — apoptotic index; PPSI — immunoreactivity score (see Methods).

**Figure 1.** Apoptotic activity in spontaneous abortions (SA) and elective terminations (ET). **A–1.** Immunohistochemical (IHC) detection of M30 in the SA group. **A–2.** IHC detection of M30 in ET group (100×). Arrows indicate M30 expression in syncytiotrophoblast. **B–1.** TUNEL assay in SA group (200×) **B–2.** TUNEL assay in ET group (100×). Arrows indicate apoptotic nuclei. **C.** Relative apoptosis levels in trophoblast tissues from SA and ET groups. ***p < 0.001 vs. ET group.**

**Table 2.** Correlation of M30 (PPSI) expression or TUNEL (AI) staining with maternal age and gravidity in spontaneous abortions and elective terminations groups

**Figure 1.** Apoptotic activity in spontaneous abortions (SA) and elective terminations (ET). **A–1.** Immunohistochemical (IHC) detection of M30 in the SA group. **A–2.** IHC detection of M30 in ET group (100×). Arrows indicate M30 expression in syncytiotrophoblast. **B–1.** TUNEL assay in SA group (200×) **B–2.** TUNEL assay in ET group (100×). Arrows indicate apoptotic nuclei. **C.** Relative apoptosis levels in trophoblast tissues from SA and ET groups. ***p < 0.001 vs. ET group.**

**Table 2.** Correlation of M30 (PPSI) expression or TUNEL (AI) staining with maternal age and gravidity in spontaneous abortions and elective terminations groups

**Syncytiotrophoblast of the SA group is shown in** Fig. 1 B1. Schematically, these observations are presented in Fig. 1C.

**M30 expression and TUNEL staining are significantly correlated with maternal age and primigravidity in spontaneous abortions**

In the control (ET) group there was no significant correlation between M30 expression and age (Spearman’s ρ = 0.266, p = 0.156) or gravidity (0.73 ± 0.47 in primigravida women vs. 0.68 ± 0.48 in multigravida women, with p = 0.812), as shown in Table 2. On the contrary, in the SA (study) group, a statistically significant correlation between M30 expression and age (Spearman’s ρ = 0.458, with p = 0.002) was observed. In addition, the M30 expression was enhanced in primigravida women compared to multigravida (4.63 ± 1.33 vs. 3.80 ± 1.08, with p = 0.036).

Similarly, in the control (ET) group there was no significant correlation between TUNEL expression and age (Spearman’s ρ = −0.005, with p = 0.981) or gravidity (0.73 ± 0.47 in primigravida women vs. 0.79 ± 0.42 in multigravida women, with p = 0.710), as shown in Table 2. On the other hand, in the SA (study) group, a statistically significant positive correlation between TUNEL expression and age (Spearman’s ρ = 0.459,
Determination of the diagnostic accuracy of M30 expression and TUNEL staining in spontaneous abortions

In order to evaluate the diagnostic accuracy of the M30 marker (PPSI) in spontaneous abortions, a ROC curve was plotted and the area under the curve was calculated as 0.983 (95% AUC = 0.956–1,000, p < 0.001) (Fig. 2). ROC curve analysis determined the cut-off value between the spontaneous abortions group and the control group, as 1.5, with the specificity of 100.0% and sensitivity of 95.2%. The positive (PPV) and negative (NPV) prognostic values were 100% and 93.8%, respectively. The overall agreement of the classification of SA specimens was 97.2%, while Cohen’s kappa coefficient confirmed these results (Cohen’s kappa = 0.943, p < 0.001). Table 3 sums up the characteristics of the diagnostic accuracy of M30 in spontaneous abortions.

A similar analysis was followed for the evaluation of the diagnostic accuracy of TUNEL assay (AI) for spontaneous abortions. Therefore, a ROC curve was plotted and the area under the curve was calculated as 0.982 (95% AUC 0.952–1,000, p < 0.001) (Fig. 3). ROC curve analysis determined the cut-off value between the spontaneous abortions group and the control group, as 1.5, with the specificity of 100.0% and sensitivity of 95.2%. The positive (PPV) and negative (NPV) prognostic values were 100% and 93.8%, respectively. The overall agreement of the classification of SA specimens was 97.2%, while Cohen’s kappa coefficient confirmed these results (Cohen’s kappa = 0.943, p < 0.001). Table 3 sums up the characteristics of the diagnostic accuracy of TUNEL in spontaneous abortions.
Trophoblast apoptosis in spontaneous abortions

According to the TUNEL values (AI), with the initial classification/diagnosis was 97.2%, while Cohen’s kappa coefficient confirmed these results (Cohen’s kappa = 0.943, p < 0.001) (Table 3).

Furthermore, both age and gravidity were tested as potentially confounding factors related to the expression of either M30 or TUNEL assay. To this end, ANCOVA demonstrated that the difference in M30 expression between SA and ET group remained statistically significant after adjusting for both factors (adjusted mean value ± S.E.: 0.89 ± 0.18 in ET group vs. 4.20 ± 0.15 in SA specimens, with p < 0.01). A similar conclusion was deducted regarding expression, where the adjusted mean value ± S.E. was calculated as 0.97 ± 0.21 for ET vs. 3.90 ± 0.18 for SA specimens (p < 0.01).

Discussion

Spontaneous abortion is a common pregnancy complication. Although SA has been associated with various factors, almost 55% of cases remain unexplained [12]. On the other hand, it is widely accepted that apoptosis has a key role in late-term pregnancy disorders, related to placenta development such as pre-eclampsia and intrauterine growth restriction [8, 10]. We believe that the role of apoptosis in the trophoblast is a factor implicated in the pathogenesis of spontaneous abortions. Thus, the present study focuses on the significance of the apoptotic mechanism during the first trimester of pregnancy and attempts to positively correlate it with increased spontaneous abortion incidences. To this end, we evaluated the relative expression of two apoptotic markers in trophoblast tissues originating from either spontaneous abortions or elective terminations.

This study demonstrated a significantly increased apoptotic expression in spontaneous abortions compared to the elective terminations group. This finding is in accordance with previous reports [13–15]. Sun et al. suggested that apoptosis and trophoblast proliferation appear in a balance [16]. Consequently, in pathological cases such as spontaneous abortions, higher apoptotic activity is observed, as demonstrated in this study [16].

Furthermore, using two independent apoptotic markers, the M30 marker, and TUNEL assay, we confirmed a previous observation that apoptotic cells are mainly detected in syncytiotrophoblast, in spontaneous abortions [17]. Thus, we believe that exaggerated apoptotic phenomena at this particular placental site, are significantly correlated with spontaneous abortion incidences, as syncytiotrophoblast is the site involving the interface of maternal and fetal tissues.

In addition, our statistical analysis revealed a positive correlation between maternal age and SA incidences, although there was no statistically significant difference between recurrent spontaneous abortion and control healthy groups in another study that concerned, though, women of younger ages [16]. However, we should take into account that many young women proceed in pregnancy termination [18]. Although our results concerning maternal age and its correlation with increased SA and apoptosis could be regarded as preliminary, further investigation towards this direction is required.

Moreover, according to our analysis, a correlation between gravidity and SA incidences was demonstrated. In fact, spontaneous abortions were positively related to primigravidity, in accordance with other larger studies [19, 20]. Interestingly, enhanced M30 expression was significantly associated with maternal age and primigravidity only in spontaneous abortions, unlike the ET control group. A similar statistically significant correlation of TUNEL expression with maternal age and primigravidity was established only in specimens derived from spontaneous abortions. To our knowledge, this is the first report that correlates these three parameters, i.e., apoptosis, maternal age, and primigravidity in spontaneous abortions.

It’s worth mentioning that our statistical analysis excluded the possibility of M30 and TUNEL acting as confounding factors. As these factors are two well-studied apoptotic markers in pre-eclampsia of late pregnancy [21], in the present study we suggest a role for these markers in spontaneous abortions during the first trimester, indicating a possible common pathophysiological base, either during the first trimester resulting in increased SA incidences or during late pregnancy and demonstrated as pre-eclampsia.

A novel finding resulting from our study is the establishment of M30 and TUNEL assay diagnostic accuracy in spontaneous abortion cases. Both markers showed very high specificity and sensitivity, with positive and negative prognostic values of 100%. Moreover, the overall agreement of the classification of spontaneous abortions specimens according to the M30 (PPSI) and TUNEL assay (AI) values with the initial classification/diagnosis was extremely high. Other studies have also investigated the role of placental apoptosis in pregnancy complications [22–27]. However, to our knowledge, this is the first study to evaluate the diagnostic accuracy of the widely used apoptotic markers, M30 and TUNEL assay, in human spontaneous abortions.

References

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