Maternal Smoking, Intrauterine Growth Restriction, and Placental Apoptosis

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ABSTRACT

Pregnant women who smoke are at greater risk of delivering a growth-restricted infant than nonsmoking mothers. We wanted to see if apoptosis could be involved in the mechanisms behind smoke-induced growth restriction, and our aim was to compare apoptosis in the placenta of smoking mothers giving birth to growth-restricted infants and nonsmoking mothers with infants of appropriate weight. The project was conducted at the Magee—Womens Hospital and Magee—Womens Research Institute, University of Pittsburgh, PA. Histological sections from 20 placentas were selected from smoking mothers who had given birth to small-for-gestational-age infants (birth weight ≤ 2 SD). The controls were gestational-age matched nonsmoking mothers with infants having appropriate-for-gestational-age weight. The TUNEL method was used to demonstrate DNA fragmentation in nuclei, and a monoclonal antibody M30, specific for a neo-epitope on cytokeratin 18, was used to identify apoptotic epithelial cells. The positive nuclei (TUNEL) and positive cells (M30-positive cytoplasm) were counted blindly both in villous tissue and in decidual/basal plate tissue. M30-positive cells in

villous tissues were significantly increased in placentas from smoking mothers compared to nonsmoking mothers. When evaluated by the TUNEL method, the difference between the two groups of women was not significant. Our study shows that apoptosis was increased in the placentas of smoking mothers with growth-restricted infants. The difference between the two groups was mainly in the syncytiotrophoblast layer and in connection with perivillous fibrin deposition. Cigarette smoke with reduction in blood flow has previously been shown to increase apoptosis, and it is possible that this could be one of the mechanisms playing a role in the growth restriction.

Key words: apoptosis, growth restriction, placenta, pregnancy, smoking

INTRODUCTION

Pregnant women who smoke are at greater risk of giving birth to a growth-restricted infant, than nonsmoking women [1]. They present a higher rate of perinatal deaths, in addition to an increased risk of spontaneous abortion and preterm delivery [2,3]. Women who smoke are also at risk for placenta previa, abruptio placentae, and premature rupture of the membranes [1]. Babies of smokers

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weigh on the average 150–250 g less than non-smokers' babies. This smoking-related weight reduction is independent of other maternal and infant factors that influence birth weight [1]. The mechanisms leading to intrauterine growth restriction (IUGR) in infants of smoking mothers are not clear. Nicotine and cotinine, a metabolic product of nicotine, both cross the placenta, and a direct toxic action of the components of cigarette smoke on the fetus is one of the most accepted theories explaining the adverse effects of smoking, though not clearly understood [3]. Nicotine is also a potent vasoconstrictor and it is believed that smoking affects intrauterine vessels, with an ensuing constriction contributing to diminished blood flow to the placenta [4]. A direct correlation between fetal oxygen consumption and birth weight has been demonstrated in experimental studies [5]. The fetal growth restriction associated with maternal smoking might therefore be related to chronic hypoxia.

Hypoxia has been hypothesized to induce apoptosis [6]. At the same time, hypoxia has been shown to induce resistance to apoptosis in several cell types, and a significant decrease in neutrophil apoptosis under hypoxic conditions has been demonstrated [7]. Both nicotine and cotinine have been related to the inhibition of apoptosis in both normal and tumor cell cultures [8,9]. Maternal smoking also appears to reduce apoptosis in villous trophoblasts [3], though studies have also shown that nicotine has little effect on cytotrophoblast apoptosis [10].

The proliferation rate of cytotrophoblasts is inversely related to oxygen tension [11]. Since hypoxia has been suggested as the main pathogenic factor in smoking, it is remarkable that no increase in proliferation rate has been observed with maternal smoking [12]. In placentas from mothers who smoked during pregnancy, there is a decreased number of cytotrophoblasts. Recent research has demonstrated that nicotine has a significant negative impact on mitosis, reducing the number of cytotrophoblasts [10]. The placental transfer of amino acids is also impaired in smoking mothers, which may have implications for intrauterine growth restriction [13].

Extensive morphological changes have been observed in placentas of smoking mothers, and oxygenation and the passage of nutrients may be

limited by these changes. Since oxygen tension is associated with apoptosis [14], it is reasonable to hypothesize an association between smoking, breaks in the trophoblast covering of the villi with deposition of fibrin, and placental apoptosis [15,16].

Few studies on maternal smoking and placental apoptosis have been conducted [3,10,17]. Some controversy exists as to the effect of smoking on apoptosis [3,9, 10, 17,18–20]. Maternal smoking and fetal growth has been extensively studied [1, 2, 21,22–24], though the correlation between intrauterine growth-retarded infants in smoking mothers and placental apoptosis has not been elucidated.

In order to see if intrauterine growth restriction caused by maternal smoking influenced placental apoptosis, we conducted a retrospective study comparing apoptosis in the placenta of smoking mothers giving birth to small-for-gestational-age (SGA) infants and nonsmoking mothers with infants of appropriate-for-gestational-age (AGA) weight.

METHODS

The project was conducted at the Magee—Womens Hospital and Magee—Womens Research Institute, University of Pittsburgh. The Institutional Review Board at the hospital approved the project.

Tissue specimens

Cases and controls were selected using the following criteria:

Cases

Twenty placentas from smoking mothers having given birth to an infant with SGA birth weight ≤ 2 SD, as assessed by standard weight percentiles [25].

Controls

Twenty placentas from nonsmoking mothers having given birth to an infant with AGA (10th–90th percentile) birth weight.

We could have used other control groups, for example, smokers with normal-weight infants or nonsmokers with growth-restricted infants. In the first category, placental changes are less likely to be expected, and in the second, other reasons for the growth restriction could confound the results.

Table 1. Clinical information

Smokers with SGA infants						Nonsmokers with AGA infants			
No.	Parity	Clinical information	Smoking (n = cig.)	GA (wk)	Birth weight (g)	Parity	Clinical information	GA (wk)	Birth weight (g)
1	G1P0	Severe IUGR	20	33.4	1164	G5P3	Placenta previae	33.0	2412
2	G3P2	IUGR	20	38.0	1915	G3P2	Normal pregnancy	37.2	3108
3	G3P2	Asthma	10	41.0	2505	G3P2	Placenta previae	41.1	3833
4	G5P4	IUGR	+	36.0	1406	G3P1	PROM	36.0	2398
5	G2P1	Tetralogy of Fallot	20	37.0	2359	G3P2	Placenta previae	37.0	2844
6	G3P1	IUGR, Rh-	20	39.0	2727	G2P1	Normal pregnancy	39.0	3089
7	G1P0	IUGR, asthma	20	38.0	2130	G3P1	Placenta previae	37.1	3461
8	G3P2	IUGR, asthma	15	36.5	1743	G1P0	PROM	35.0	2395
9	G2P1	IUGR	20	38.6	2361	G2P0	Maternal fever	40.1	3120
10	G2P0	IUGR	8	38.0	2351	G2P0	Normal pregnancy	38.6	2951
11	G3P2	IUGR, Rh-, drug abuse	15	35.6	1728	G2P1	Fetal distress	35.0	2345
12	G1P0	PROM, fetal distress	20	29.1	668	G4P2	Cerclage PROM	28.6	1462
13	G1P0	Drug abuse	40	38.4	2228	G5P2	Normal pregnancy	39.0	3640
14	G2P0	IUGR	10	38.0	2183	G1P0	Normal pregnancy	38.0	3246
15	G2P1	IUGR	40	38.0	2138	G2P1	PROM	38.0	3510
16	G2P1	IUGR, fetal distress	15	35.0	1212	G2P1	Placenta previae	35.0	2276
17	G1P0	IUGR	+	37.3	2196	G2P1	Placenta previae	37.0	3012
18	G1P0	IUGR	10	37.0	2257	G1P0	Placenta previae	37.0	2979
19	G2P0	Severe IUGR	10	25.1	791	G2P0	Placenta previae	26.0	850
20	G2P0	Asthma	20	36.4	1626	G3P2	Placenta previae	36.4	3093

SGA, small-for-gestational-age; AGA, appropriate-for-gestational-age; G, gravida; P, para; cig., cigarettes; GA, gestational age; wk, weeks; PROM, premature rupture of the membranes; IUGR, intrauterine growth restriction.

Previous studies have shown that apoptosis increases towards term [26,27]. The controls and cases were therefore matched for gestational age.

Information was obtained by using the database at the Department of Pathology. All reports were from the period 1996 to 2000, and supplementary clinical information was pulled from the hospital records.

Placentas from women with preeclampsia, gestational hypertension, diabetes, infection, or any other serious illness were excluded from the study. Placentas from infants with serious or multiple developmental anomalies, and placentas with chorioamnionitis or extensive villitis, were also excluded. Clinical data on both groups are given in Table 1. The results of the morphological examination of placental tissue are shown in Table 2.

Two formalin-fixed and paraffin-embedded blocks were retrieved from each placenta, one

from the periphery and one central. Five micron-thick sections were cut from each block. In order to evaluate apoptosis, both the TUNEL method and immunohistochemistry with a M30 antibody were performed.

TUNEL method

A DNA fragmentation kit was used for the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate [dUTP] nick end labeling) staining (Oncogene Research Products, Boston, MA). The nick end labeling refers to staining of the DNA 3' hydroxy groups generated as a result of endonuclease activity at linker DNA regions between nucleosomes. The commercially given instructions were followed. TUNEL-positive nuclei stained dark brown, and when they also looked pyknotic, they were regarded as apoptotic. Sections from human lymph node were included in all

Table 2. Placental examination

Smokers with SGA infants				Nonsmokers with AGA infants		
No.	Placental weight (g)	Weight percentile	Morphological findings	Placental weight (g)	Weight percentile	Morphological findings
1	200	<10	AVM, increased perivillous fibrin 20%, increased decidual fibrin	370	25–50	NVM, mild chorionic hemosiderosis
2	220	<10	NVM, increased perivillous fibrin <5%	470	50–75	NVM, normal morphology
3	400	25–50	NVM, subchorial intervillous thrombi, intervillous fibrin	290	<10	NVM, meconium macrophages
4	260	<10	NVM, meconium macrophages	340	10–25	NVM, partial necrosis of amnion
5	275	<10	NVM, villous sclerosis and hemorrhagic endovascularitis 15%	400	25–50	NVM, subchorionic fibrin, marginal blood clot
6	410	25–50	NVM, villous sclerosis, minimal chorioamnionitis	360	10–25	NVM, retroplacental hematoma <1%, villous sclerosis <5%
7	500	75–90	NVM, subchorionic fibrinous plaques	600	>90	NVM, flattened segment involving 30% of disc
8	290	<10	Multiple infarcts 30%, recent marginal intervillous thrombus	410	50–75	NVM, mild villous edema
9	280	<10	DVM, meconium macrophages	410	25–50	NVM, normal morphology
10	320	<10	NVM, subamniotic hemorrhage, subchorionic fibrinous plaques	400	25–50	NVM, meconium pigment accumulation
11	450	50–75	NVM, mural thrombus chorionic vein, focal chorangiosis <5%	360	10–25	AVM, meconium macrophages
12	145	<10	AVM, multiple infarcts 15%, decidual vasculopathy	235	10–15	NVM, intervillous hemorrhage 10%, chorangiosis
13	290	<10	NVM, normal morphology	490	75–90	NVM, two vessels in umbilical cord
14	460	50–75	NVM, normal morphology	460	50–75	NVM, meconium macrophages
15	440	50–75	NVM, mild perivillous fibrin deposition	500	75–90	NVM, normal morphology
16	250	<10	NVM, recent central infarcts <10%	340	10–25	NVM, subchorionic fibrinous plaques
17	320	<10	NVM, focal membranous edema	600	>90	NVM, circummarginate placenta
18	375	25–50	NVM, marginal perivillous fibrin, multiple infarcts <10%	370	25–50	Intervillous thrombi, marginal blood with focal abruption
19	150	<10	AVM, villous infarctions 50%, focal placental abruption	200	25–50	NVM, maternal blood clot with focal placental abruption
20	370	25–50	NVM, fetal thrombotic vasculopathy 25%	345	10–25	NVM, normal morphology

SGA, small-for-gestational-age; AGA, appropriate-for-gestational-age; AVM, advanced villous maturation; NVM, normal villous maturation; DVM, delayed villous maturation; %, percent of affected placental volume.

batches as positive controls. Sections from placental tissue, to which addition of enzyme was replaced by distilled water, served as negative controls.

Immunostaining with the monoclonal antibody M30

The monoclonal antibody (mab) M30 (Boehringer Mannheim, Mannheim, Germany), specific for a neo-epitope on cytokeratin 18 (CK18), was used to recognize apoptotic epithelial cells [28,29]. CK 18 is cleaved by caspases early in the apoptotic cascade, thus visualizing apoptosis before DNA fragmentation detectable by the TUNEL method. Immunostaining with M30 was performed as previously described [30]. Sections from an adenocarcinoma of colon were added as positive controls [31]. M30 positivity gives a cytoplasmic staining, most often homogeneous, but sometimes more granular. Cells with pyknotic nuclei and red staining of cytoplasm were regarded as apoptotic. The TUNEL method and the mab M30 immunostaining were performed on serial slides from each placenta, and all the slides were blinded. The coded slides were selected in such a way that the gestational age-matched cases and controls were included in the same batch.

Quantification of apoptosis

TUNEL-positive nuclei and M30-positive cells were counted blindly according to the criteria given above. Twenty fields of villous tissue at 200 $\times$ magnification and 20 fields of maternal basal plate tissue at 400 $\times$ magnification were counted on each slide, though on a number of slides there was not sufficient basal plate tissue to count 20 fields. Only fields with at least 80% tissue coverage were evaluated. The localization and origin of the positive cells were taken into consideration and categorized thereafter. When TUNEL-positive nuclei and M30-positive cells in all slides were counted, the code was broken. Cells from the peripheral and central sections were added to each other so that altogether 40 fields of villous tissue from each placenta were evaluated. With M30 immunohistochemistry, the number of fields in decidual/basal plate tissue varied from 4 to 36, with 18 as the median, with interquartile ranges (IQR) corresponding to 25–75% of 12.0–24.0. For the TUNEL

method, the number of fields varied from 15 to 40, with 40 as the median, IQR 25–75% of 31.3–40. The number of M30- and TUNEL-positive cells per field was calculated, and the rate of apoptosis in villous and decidual/basal plate tissue is shown in Table 3.

Statistical analysis

It was calculated that to obtain a statistical power of 90%, study groups of 20 were needed. Sample size calculations were performed on the Number Cruncher Statistical Systems (NCSS) program (Hintze J [2001] Kaysville, UT, USA). The apoptotic rate showed a marked skewed distribution towards zero, therefore median group values were employed with corresponding IQR and application of nonparametric statistics. In order to evaluate differences in apoptotic rate between the matched groups, *P*-values were calculated by using Wilcoxon signed-rank test for paired data. For unmatched comparison, the Mann–Whitney *U*-test was applied. Statistical analyses were performed using Statistical Package for Social Services (SPSS) version 11 (SPSS for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

M30-positive cells showed either a homogenous or granular red cytoplasmic staining (Fig. 1A). Positive syncytiotrophoblasts were either located on the villous surface, as syncytial knots (Fig. 1B), or lying in connection with peri- and intervillous fibrin deposition (Fig. 1C). Like the M30 positivity, TUNEL-positive nuclei were found in the villous syncytiotrophoblast layer (Fig. 2A), in syncytial knots (Fig. 2B), and in connection with fibrin deposition (Fig. 2C).

Total cell number in fields of villous tissue ($n = 5$) was counted in order to calculate the percentage of apoptotic cells. The median number was 840, IQR: 816–833. If 840 represents the total cell number in the evaluated villous field, the total apoptotic rate in villous tissue of controls as evaluated by M30 was estimated as 0.04%, IQR: 0.02–0.07%.

Table 3 shows the number of apoptotic cells per field in various placental tissues as assessed by immunostaining with the M30 antibody. M30 positivity was significantly increased in villous tissue

Table 3. Number of apoptotic cells per field in various placental tissues as assessed by mab M30

Tissue	Group	Median ^a	25th	75th	<i>P</i> -value*
Villous tissue	Smokers	0.56	0.48	0.96	0.04
	Controls	0.35	0.18	0.63	—
Syncytiotrophoblast layer	Smokers	0.13	0.08	0.20	0.50
	Controls	0.10	0.05	0.15	—
Peri/intervillous fibrinoid	Smokers	0.38	0.16	0.73	0.17
	Controls	0.19	0.08	0.44	—
Villous stroma	Smokers	0.03	0.01	0.05	0.49
	Controls	0.04	0.01	0.07	—
Decidual/basal plate	Smokers	0.61	0.24	0.67	0.10
	Controls	0.38	0.09	0.66	—
Anchoring villi	Smokers	0.10	0.04	0.22	0.42
	Controls	0.10	0.00	0.20	—
Extravillous trophoblasts	Smokers	0.33	0.13	0.56	0.06
	Controls	0.23	0.00	0.41	—
All tissues combined	Smokers	1.10	0.79	1.41	0.10
	Controls	0.79	0.27	1.40	—

mab, monoclonal antibody.

^aMedian number of apoptotic cells per field counted with 25th and 75th percentiles.

**P*-value: Wilcoxon paired-rank correlation test of difference towards the control group.

(including syncytiotrophoblasts and peri/intervillous fibrin) in placentas from smoking mothers with SGA infants compared to nonsmoking mothers with normal infants. Generally, the M30 count was higher in the smoking group compared to the control group, though except for total villous tissue, this difference was not significant. There was no significant difference between the two groups of women with regard to apoptosis as evaluated by the TUNEL method. This pertains to all types of tissue counted: syncytiotrophoblasts, fibrin, villous stroma, anchoring villi, and extravillous trophoblasts.

In decidual/basal plate tissue, M30-positive extravillous cytotrophoblasts and TUNEL-positive nuclei were detected. Since M30 positivity occurs in epithelial cells, it was not relevant to compare the decidual cells. There was an increased number of M30-positive cells in extravillous trophoblasts ($P = 0.06$) but not in anchoring villi in smokers as compared to controls (Table 3).

Morphological alterations in the placenta were more frequent in placentas of smoking mothers (Table 2). Advanced villous maturation was described in one case in the control group and in

three cases in the smoking group. Peri/intervillous fibrin deposition or infarcts were found in 10 of the smoking cases, while in none of the control group. Five cases were described as largely normal in the smoking group, while 15 were described as normal in the control group.

DISCUSSION

Two major reasons for the intrauterine growth restriction in infants of smoking mothers have been proposed, namely toxic effects of nicotine and its metabolites, and a vasoconstrictor effect causing diminished blood flow to the placenta with fetal hypoxia [4]. The placenta retains substantial amounts of compounds from cigarette smoke, both in their native forms and in intracellular substrates [32,33]. Cotinine levels in plasma have been shown to correlate with uteroplacental vascular resistance estimated from blood flow velocity measures by Doppler [4,34].

What is then the link to apoptosis? Apoptosis, or programmed cell death, was first described by Kerr et al. [35]. It is a normal physiological form of cell death, that together with mitosis, has the function of controlling cell population [26,36]. The pro-

Figure 1. M30-positive cells. **A.** Homogenous or granular red cytoplasmic staining. **B.** Positive syncytiotrophoblasts. **C.** Positive cells in fibrin.

Figure 2. TUNEL-positive nuclei. **A.** In villous syncytiotrophoblast layer. **B.** In syncytial knots. **C.** In connection with fibrin deposition.

cess may be influenced by different stimuli, both physiological and nonphysiological [37].

Nicotine has been related to the inhibition of apoptosis in cell cultures [8,9], while hypoxia has been shown to induce apoptosis [6,38,39]. Enhancement of apoptosis by hypoxia has also been demonstrated in cultured human trophoblasts, while addition of epidermal growth factor (EGF) to the cultures lowers the level of apoptosis [39], suggesting that EGF may protect against apoptosis induced by hypoxia.

Cigarette smoke has also been shown to increase apoptosis in the gastric mucosa of rats, by reducing gastric blood flow, thus decreasing serum epidermal growth factor [19,20]. Could the same mechanisms play a role in the uterus, with a decrease in serum EGF implicated in the intrauterine growth restriction? Other studies have not shown increased apoptosis in placentas of smoking mothers [3], however, these have not taken into account whether the infants were growth restricted or not.

Increased apoptosis has been found in placentas of pregnancies complicated by fetal growth restriction [40]. We also found a significant increase of apoptosis in villous tissue in the placentas of smoking mothers who delivered a growth-restricted infant. This increase could be explained by hypoxia accentuated by reduced blood flow with decrease in EGF. Since EGF is present in amniotic fluid [41], the IUGR found in children of smoking mothers might be a direct effect of decreases in EGF levels either in serum or amniotic fluid. Nicotine has also been shown to have an inhibitory effect on early trophoblast differentiation [10,42], and the increased apoptosis may, in part, be due to a direct toxic effect of the components of cigarette smoke [3].

Apoptosis, as evaluated by the TUNEL method, was not significantly increased as compared to controls. Completely normal controls for the various gestational ages were difficult to find, and altogether nine cases had placenta previae. Some of these placentas had higher TUNEL counts than M30, which we ascribe to the fact that TUNEL staining is not specific for apoptosis, but also stains necrotic cells [43]. It is therefore important to take into account false-positive TUNEL staining. With the M30 antibody, there was little nonspecific staining present, and we recommend

the use of this antibody when studying apoptosis in CK18-positive epithelial cells.

In smoking mothers, the placental/fetal ratio is increased and morphological alterations have been demonstrated [42,44]. In our study, infarcts or peri/intervillous fibrin deposition were registered in 50% of cases in the smoking group, with none in the control group. Fibrinoid deposition during gestation occurs at sites of trophoblast discontinuity, it is nonrandom and influenced by the quality of vascular perfusion [16,45, 46]. Smoking during pregnancy affects placental blood flow [47,48], therefore, the morphological findings in placentas of smoking mothers do not seem to be haphazard, but related to the effects of cigarette smoke.

During the first trimester, focal defects in floating villi with absence of cytotrophoblast stem cells have been demonstrated, together with an increase in the number of cytotrophoblast columns that fail to reach the uterus or degenerate in the intervillous space, implying a reduction in the number of anchoring villi [10]. A reduced number of Ki-67-positive cytotrophoblasts means that fewer cells are in the S-phase causing a premature depletion of cytotrophoblast stem cell population [10]. We can thus conclude that the cytotrophoblast population in placentas of smoking mothers is reduced. When apoptosis among these cytotrophoblasts is increased, this will further diminish blood flow and oxygenation potential over the fetal-maternal membrane, also decreasing EGF and escalating the nutritional defects to the fetus.

CONCLUSION

Our study shows that apoptosis in fetal villous tissue is increased in the placentas of smoking mothers with SGA infants compared to gestation-matched controls with AGA infants and mothers who do not smoke. Increased peri/intervillous fibrin deposition was found in the smoking group, but not in the control group. The role of hypoxia in the induction of apoptosis has previously been demonstrated. Apoptosis in syncytiotrophoblasts causes discontinuities in the trophoblast layer and deposition of fibrin-type fibrinoid, which further reduces factors implicated in growth.

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