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Proliferation of villous trophoblast of the human placenta in normal and abnormal pregnancies

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Summary. The proliferation of villous trophoblast in the human placenta was estimated throughout normal gestation and in term placentae from preeclamptic and smoking mothers by two different methods. These were: 1) labeling of DNA producing cells by bromodeoxyuridine (BrdU) followed by immunohistochemistry using a monoclonal anti-BrdU antibody, and 2) immunohistochemical identification of all proliferating cells by the monoclonal antibody Ki67. Both methods revealed comparable results. In uncomplicated pregnancies there was a remarkable decrease in the labeling indices from early gestation to term. This was the result of a diminution of the number of Langhans' cells, although the cell division rate within the Langhans' cell layer remained nearly constant throughout gestation. A prolongation of the cell cycle in the cytotrophoblast cells at term was indicated by an increase in the Ki67/BrdU ratio. Compared with normal term placentae, there was an increase in the trophoblast proliferation rate in preeclampsia, but not in placentae from smoking mothers. Moreover, the number of Langhans' cells was diminished in placentae from smokers. The results indicate that there are different pathogenetic mechanisms of placental impairment in preeclampsia and in maternal smoking. In preeclampsia an injury to the syncytiotrophoblast seems to lead to a repair hyperplasia of the cytotrophoblast, whereas in maternal smoking, there seems to be a direct toxic effect on the cytotrophoblastic cells.

Key words: Placenta – Trophoblast – Proliferation – Preeclampsia – Smoking

Introduction

Chorionic villi of the human placenta are covered by two trophoblastic layers, the outer syncytiotrophoblast and the inner cytotrophoblast, the so-called Langhans' cells. The syncytium has irreversibly lost its ability to divide and grows by fusion of cytotrophoblast cells. This process is of great importance in the growth of the villous tree and in the ability of the trophoblast to regenerate under pathological conditions. In accordance with the rapid growth of the placenta in the first trimester, the layer of Langhans' cells is nearly complete at that time. In contrast, there are only a few Langhans' cells left at term. It has been reported that there is an increase in the number of Langhans' cells under various pathological conditions, such as preeclamptic toxemia and diabetes mellitus (Wigglesworth 1962; Fox 1964; Kaufmann et al. 1977; Jones and Fox 1980, 1981; Soma et al. 1982; Teasdale 1985). However, only a few histoautoradiographic studies of trophoblast proliferation in pathological placentae have been published and the results have been discordant (Krauer 1970b; Kaltenbach et al. 1974; Hustin et al. 1984; Toussaint 1989).

Therefore, we have investigated the effect of preeclampsia and smoking during pregnancy on trophoblast proliferation and have compared the results with trophoblast development during normal gestation. Two different immunohistochemical methods, i.e. the detection of the DNA precursor bromodeoxyuridine (BrdU) after a short term tissue culture and the labeling of proliferating cells by the Ki67 antibody, were used in an attempt to gain insights into pathogenetic mechanisms following placental injury under such pathological conditions. Labeling of different parts of the cell cycle by the two methods allows some conclusions to be drawn regarding possible changes within the cell cycle.

Material and methods

Placentae: A total of 80 placentae were examined by the BrdU and 57 by the Ki67 method; 16 of these placentae were studied by both methods. The placentae were from uncomplicated pregnancies (controls), from women suffering from preeclampsia and from women who smoked during pregnancy as shown in detail in Table 1. In the control cases there was no history of smoking

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Table 1. Details of the placentae studied

	No. of placentae studied by		Gestational age (weeks)	
	BrdU	Ki67		
Controls	14	9	6–23	Elective abortion
		3	16–22	Spontaneous abortion
	5	1	31–36	Premature delivery
	4	5	37–42	Caesarean section
	32	21	37–42	Spontaneous delivery
Preeclampsia	11	9	37–42	Mild 6/3 Moderate 5/5 Severe 0/1
Maternal Smokers	14	9	37–42	Spontaneous delivery (>10 cigarettes/day)

or disease during pregnancy. A few placentae obtained during the beginning of the second half of gestation were also included in the control group of uncomplicated pregnancies to get some information on trophoblast proliferation in this phase of gestation. These placentae were from patients suffering spontaneous abortions or premature deliveries, but in whom there were no other clinical abnormalities. All control placentae revealed a histologic structure in accordance with their gestational age. In the pathological groups, a diagnosis of preeclampsia was based on a blood pressure of 140 mm systolic or 90 mm diastolic, or a daily proteinuria of more than 300 mg/l. The cases were subclassified into three degrees of severity using the "Gestose-Index" (Gille 1986). Smoking mothers were defined as women who had smoked ten or more cigarettes a day during pregnancy according to their medical history. Patients with other maternal diseases were excluded from the three groups (controls, preeclampsia, maternal smoking).

Methods. Bromodeoxyuridine (BrdU). The incorporation of bromodeoxyuridine (BrdU), an analogue of thymidine, into the nuclei of DNA-producing cells was performed in short term tissue culture. The incorporated DNA precursor was detected immunohistochemically by a monoclonal anti-BrdU antibody (Fig. 1).

Ki67. The growth fraction was determined using the monoclonal antibody Ki67 which reacts with the nuclei of all cells in the active part of the cell cycle, i.e. G_1, S, G_2 and mitosis, but not with G_0 cells (Gerdes et al. 1984) (Fig. 2).

Tissue sampling and processing. Most of the placentae were obtained within 30 min and a few within 45 min after delivery. Preliminary tests revealed that the Ki67 labeling index remained unchanged up to 60 min. Two or more fragments of villous tissue from each placenta, measuring 0.5 to 1.0 cm^3, were collected, avoiding marginal and subchorionic parts of the placenta.

For the BrdU method the tissue was washed in three changes of phosphate-buffered saline (PBS) and then incubated at 37° C for 3 h in medium 199 (Biochrom, Berlin, FRG) supplemented with 20% fetal calf serum containing equimolar amounts of 5-bromo-2′-deoxyuridine and 5-fluoro-2′-deoxyuridine (final concentration 10 µg/ml) and 2-deoxyuridine (final concentration 8 µg/ml) (all purchased from Sigma, St. Louis, Mo, USA). The relatively long incubation time had been chosen to ensure a sufficient diffusion of BrdU into the villi. At the end of the incubation period the tissue was washed again three times in PBS, fixed in 70% ethanol for 12 h, dehydrated through alcohols and xylene and embedded in paraffin wax. Deparaffinized sections were incubated in 7.5% H_2O_2 for 5 min to inhibit endogenous peroxidase activity. After incubation in 2 N HCl for 30 min to denaturate DNA, the slides were washed twice in 0.01 M Borax buffer, pH 8.5, and once in PBS.

For the detection of proliferating trophoblast cells by the Ki67 antibody the tissue blocks were snap-frozen in liquid nitrogen and stored at −80° C. Cryostat sections of about 5 µm were air-dried overnight and subsequently fixed in acetone for 10 min.

After incubation with the anti-BrdU monoclonal antibody (1:50, clone B 44; Becton Dickinson, Rutherford, NJ, USA) or the Ki67 antibody (1:25; Dakopatts, Copenhagen, Denmark), respectively, for 30 min at room temperature, immunohistochemical staining was carried out using the Avidin-Biotin-Complex (ABC) method. Biotin-labeled rabbit anti-mouse-immunoglobuline antibody (1:250) and Avidin-Biotin-Complex were obtained from Dakopatts, Copenhagen, Denmark. The peroxidase reaction was developed with 0.01% 3-amino-9-aethylcarbazole (Sigma, St. Louis, Mo, USA) and 0.01% H_2O_2 in barbital buffer, pH 7.4. Sections were counterstained lightly with hematoxylin. Sections (5 µm) of adjacent blocks of formalin-fixed and paraffin-embedded tissue from the same placenta were taken to evaluate the population density of Langhans' cells.

Immunohistochemical staining was controlled by: 1) incubation with the secondary antibodies only, and 2) developing the peroxidase only (endogenous peroxidase). Blocking of endogenous peroxidase was omitted in the cryostat sections, so that there was a weak reaction in Hofbauer cells and granulocytes. These reactions were ignored in assessing the results.

Morphometry. For the estimation of the trophoblast labeling indices, labeled trophoblast nuclei were counted over the whole area of a square grid consisting of 25 squares at a final magnification of 160×. To reduce the labour of counting without impairing its accuracy, the unlabeled nuclei of the syncytio- and cytotrophoblast were counted in only two of the 25 squares. Thus a total of 30 randomly selected fields for each section were analysed.

The labeling indices were related to the whole trophoblast including the syncytium, as in former studies, to make comparisons possible. The relative frequency of Langhans' cells per trophoblast area was estimated on 5 µm-thick sections by counting Langhans' cells falling on the whole area of a 25 point grid of 450 × 450 µm, and determining the trophoblast area in the same field by using the point counting method (Aherne and Dunnill 1982). A total of 30 fields per tissue block were taken at random. The results were converted to a trophoblast area unit of 10,000 μm^2.

To determine the proliferation rate of the Langhans' cells only, BrdU and Ki67 labeling indices in five placentae obtained during the first trimester and in five term placentae were determined counting 1000 and 500 Langhans' cells, respectively, in randomly selected fields.

Statistics. The distribution of the mean labeling indices of the placentae of each group are depicted in box plots (Sachs 1988). The Wilcoxon U test was used for statistical evaluation.

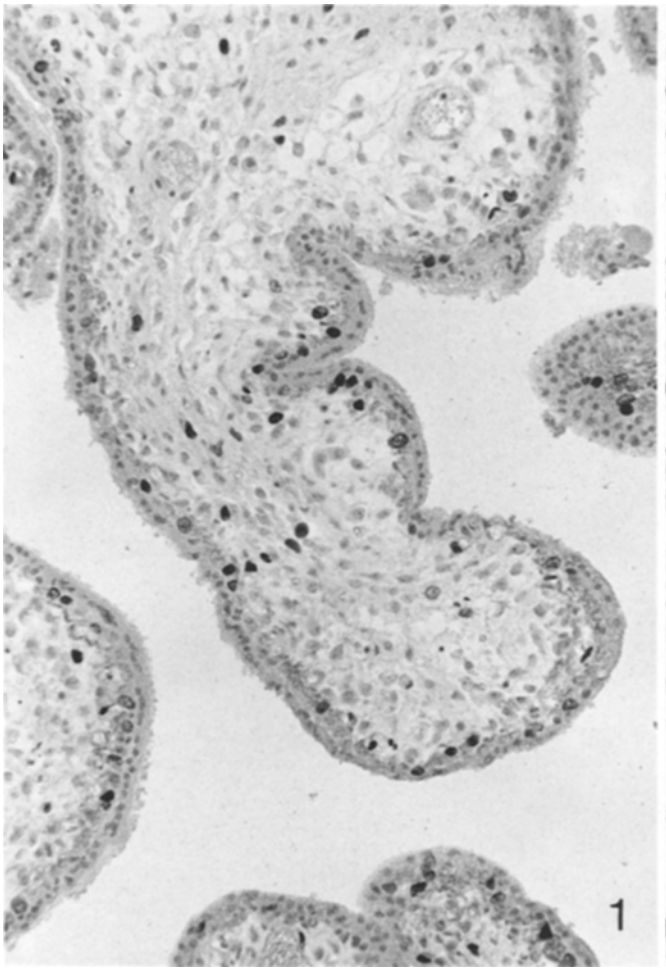


Fig. 1. Immunohistochemical localization of bromodeoxyuridine (BrdU) in paraffin sections. Positive reaction (*dark*) in cytotrophoblastic nuclei and stromal cells which synthesized DNA during the BrdU incubation period of 3 h. Human placenta at 8 weeks of gestation. (×160)

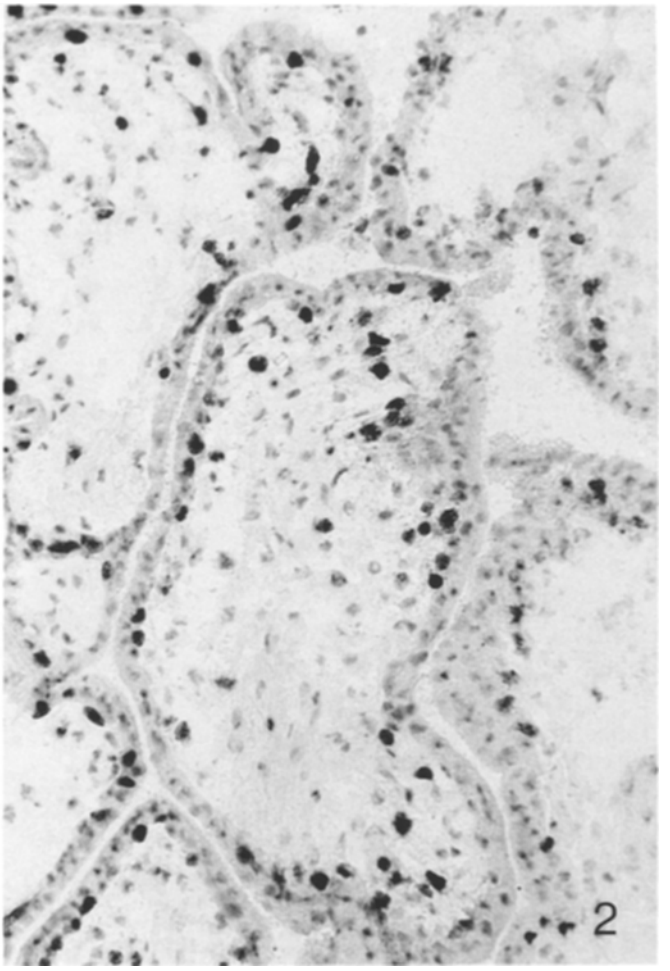


Fig. 2. Immunohistochemical localization of a proliferation-associated antigen in a frozen section using the Ki67 antibody. Positive reaction in the nuclei of all proliferating cells. Human placenta at 10 weeks of gestation. (×160)

Results

The two methods, i.e. the BrdU- and Ki67-labeling indices, used to study the proliferation of the villous trophoblast revealed comparable changes in the course of normal gestation as well as in preeclampsia and maternal smoking. In placentae from uncomplicated pregnancies, there was a marked decrease in the mean labeling indices from first trimester to term from 32.3‰ to 6.9‰ (−79%) in the BrdU group and from 45.2‰ to 15.2‰ (−66%) in the Ki67 group (Fig. 3). Compared with previous investigations using ^{3}H-thymidine histoautoradiography (Krauer 1970a; Weinberg et al. 1970; Kaltenbach et al. 1974; Hustin et al. 1984) the BrdU indices or our study are somewhat lower, possibly because of the lower sensitivity of BrdU immunohistochemistry.

As BrdU labels only cells within the S phase during the incubation period, whereas the Ki67 antibody labels all cycling cells, it is possible to estimate the proportion of the length of total cell cycle to S phase by calculating a Ki67/BrdU ratio. In uncomplicated pregnancies this Ki67/BrdU ratio showed a slight increase at term. Including only those placentae from which tissue from immediately adjacent areas could be examined by both methods, the mean Ki67/BrdU ratio rose from 1.2 in the first trimester to 3.2 at term (Table 2).

Besides these BrdU- and Ki67-indices related to the whole trophoblast, including the syncytiotrophoblast, in some placentae a labeling index of the Langhans' cells only was determined to assess whether the reduction in the proliferation rate from first trimester to term is caused solely by the decrease in the number of Langhans' cells in mature placentae. Using BrdU labeling, this Langhans' cell index showed no appreciable change during gestation. On the other hand, the Langhans' cell Ki67 index was slightly elevated at term (Fig. 4). Assuming a constant S phase duration, these findings indicate an unchanged cell division rate within the diminishing Langhans' cell population and a slightly prolonged cell cycle of the Langhans' cells at term.

At term (37 to 42 weeks of gestation) no relation was found between the labeling index of the trophoblast on the one hand and the weight of placenta or child on the other hand.

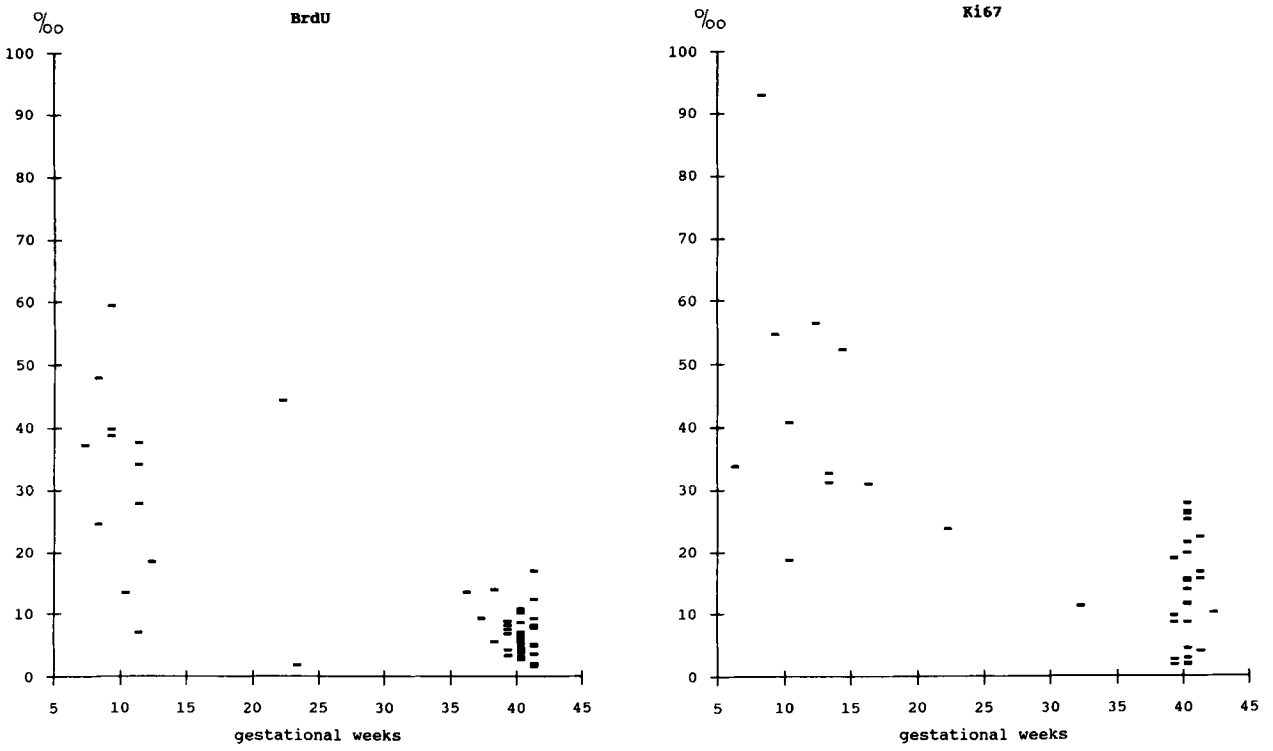


Fig. 3. Gestational age dependent change in the BrdU- (*left; n*=55) and Ki67- (*right; n*=38) labeling index of the villous trophoblast. Placentae from uncomplicated pregnancies (control group)

Table 2. Ki67/BrdU ratio. Included are placentae only, Ki67 and BrdU indices of which have been evaluated in immediately adjacent areas of the same placenta

	Gestational age (weeks)	Ki67/BrdU ratio	Mean ±SD
Abruptio	10	1.5	
	11	1.6	1.2±0.6
	12	0.6	
Premature delivery	33	2.6	–
Spontaneous delivery	38	2.3	
	40	2.2	
	41	4.7	3.2±1.2
	41	3.7	
Preeclampsia	32	1.7	–
	36	2.7	
	38	2.7	
	38	2.3	2.1±0.6
	39	1.3	
	40	1.8	
Maternal Smokers	40	5.9	
	41	1.0	–

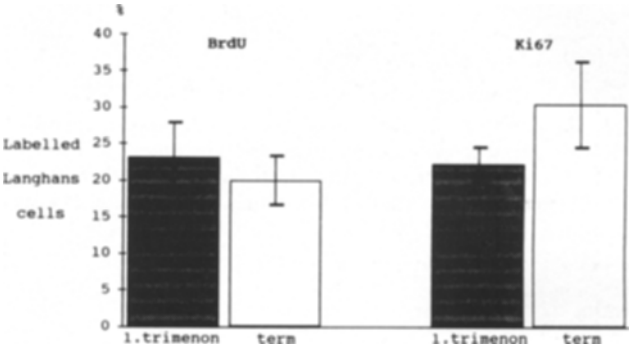
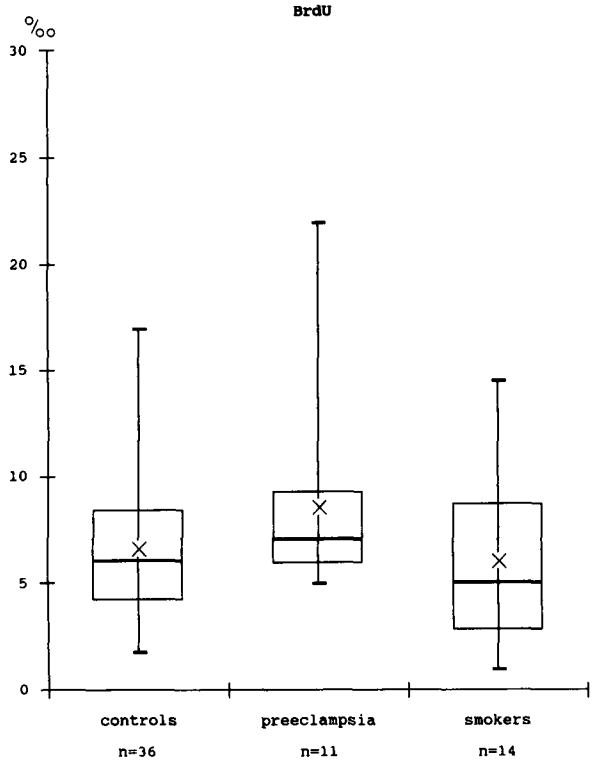


Fig. 4. BrdU and Ki67 labeling indices of the cytotrophoblastic layer (Langhans' cells) comparing normal first trimester and term placentae. Mean values ±SD, (*each group n*=5)

In comparison with normal term placentae there was a slight increase in the mean labeling indices in the preeclampsia group, using both Ki67 and BrdU. However, the differences were statistically significant only with respect to the Ki67 index ($P<0.02$). In contrast, term placentae from smoking women did not show any changes in the mean indices using Ki67 or BrdU (Fig. 5). The estimation of the frequency of Langhans' cells within a trophoblast area unit, however, showed a slight decrease in the placentae of smoking mothers ($P=0.05$), compared with normal term placentae, whereas in placentae from preeclamptic women there was no definite change (Fig. 6).

No relation could be found between the degree of preeclampsia or the number of cigarettes smoked daily and the mean labeling indices. However, in these subgroups the number of cases was small. There was a wide variation in the Ki67/BrdU ratio in the preeclampsia and maternal smoking groups compared with the controls, and in some the ratio was remarkably low (<2) (Table 2).



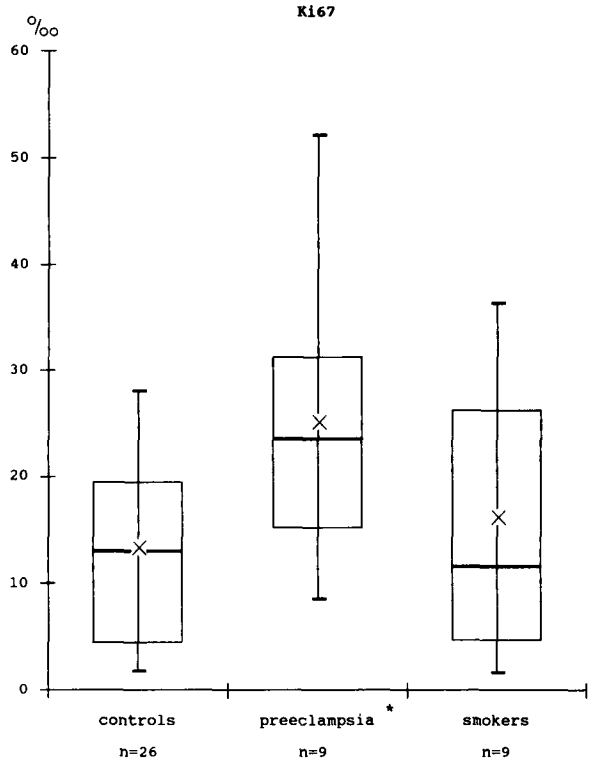


Fig. 5. BrdU- (*left*) and Ki67- (*right*) labeling indices of the villous trophoblast of term placentae (37 to 42 weeks of gestation) from uncomplicated pregnancies (controls), preeclampsia and maternal smokers shown in box plots. The boxes contain 50% of the values. Vertical lines extend to the maximum and minimum measured values. Horizontal lines represent the median, crossed lines the mean values (* $P<0.02$)

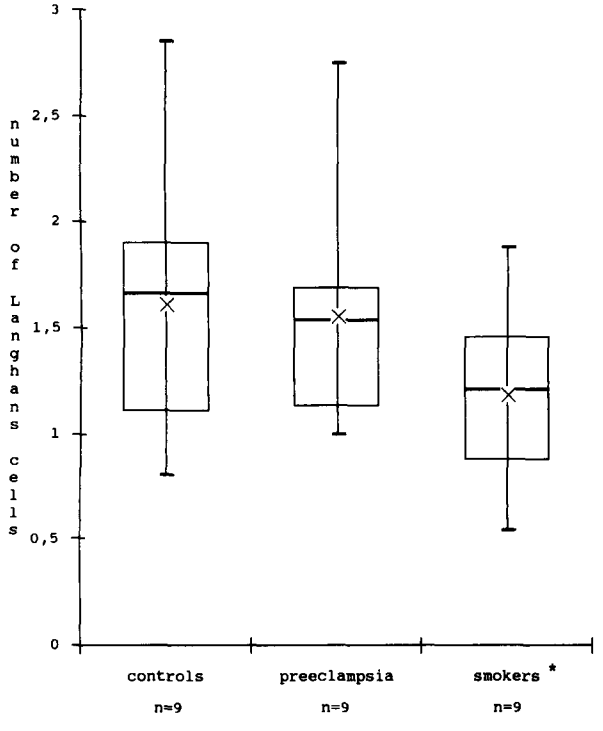


Fig. 6. Relative frequency of Langhans' cells per trophoblast area unit (10,000 μm^2) in term placentae from uncomplicated pregnancies (controls), preeclampsia and maternal smokers shown in box plots. The boxes contain 50% of the values. Vertical lines extend to the maximum and minimum measured values. Horizontal lines represent the median, crossed lines the mean values (* $P=0.05$)

Discussion

The trophoblast has to fulfill many functions in metabolism and in the maintenance of pregnancy. The increasing demands of the developing fetus are reflected in changes of trophoblastic structure. Considering the gain in placental weight and the development of the histological structure of the villous tree throughout gestation, it might be concluded that proliferation is more important in the first half of gestation, while differentiation of the chorionic villous tree is more relevant in the second half. Thus, in late gestation, placental function is further increased in regard to the rapid fetal growth in spite of the reduced rate of placental weight gain. Accordingly, our own results, as well as that of some autoradiographic studies of trophoblast proliferation during normal gestation, demonstrate a marked decrease in proliferation rate from early gestation to term (Krauer 1970a; Weinberg et al. 1970; Kaltenbach et al. 1974; Hustin et al. 1984; Pfeiffer-Ohlsson et al. 1984). Winick and coworkers (1967) have shown that the total DNA content of the placenta increases only until about 1 month prior to term. However, the detection of DNA-producing cells by morphological methods demonstrates that trophoblast proliferation occurs to a small extent even in mature placentae. Nevertheless, it is unlikely that this minor trophoblastic proliferation at term is important in placental growth. Rather, it seems that this proliferation, found exclusively in the basal trophoblastic

layer, the Langhans' cells, is necessary for the regeneration of the syncytium, the functionally differentiated part of the trophoblast. In general, there are various ways of regulating trophoblast proliferation, including changing the number of potentially or actually proliferating cells, or changing the length of the cell cycle.

By labeling different parts within the cell cycle by the two methods used in this study, Ki67 and BrdU, it is possible to get some information about changes within the cell cycle. The antigen labeled by the Ki67 antibody is expressed during the whole cell cycle, with the exception of a small part of the G_1 phase in cells returning from G_0 (Gerdes et al. 1984). BrdU, like ^{3}H-thymidine, used in autoradiographic studies, labels only cells within the S phase during the incubation period. The remarkable increase in the Ki67/BrdU ratio from early gestation to term indicates a change in the relationship between total cycle time and S phase duration. Within the cell cycle, the length of the G_1 phase is particularly variable. The S phase is rather constant, but it can be shortened, for instance, in very early embryos (Pardee 1989; Laskey et al. 1989). Thus, it seems more likely that the low Ki67/BrdU ratio in the first trimester found in this study is the result of a short G_1 phase rather than a prolonged S phase. In autoradiographic studies by Gerbie et al. (1968) using first trimester placentae, a cell cycle time of 15 h and a S phase duration of about 5–6 h was calculated. Taking into account a BrdU incubation time of 3 h as in this study, a Ki67/BrdU ratio (corresponding to the ratio of total cell cycle time to S phase duration plus incubation time) of less than 2 from these results could be estimated, which is in agreement with our own data.

Another question is whether the reduced proliferation rate of the trophoblast at term, estimated by labeling indices related to the whole trophoblast, is brought about mainly by diminishing the number of Langhans' cells, which drops to about half that in the 8th week of gestation (Kaufmann 1981), or whether the actual proliferative activity of the Langhans' cells themselves is reduced. To answer this question a labeling index related to the Langhans' cells only was determined. This evaluation revealed a nearly constant portion of BrdU-labeled S phase cells throughout gestation. In contrast, the portion of Ki67-positive Langhans' cells increased slightly at term (Fig. 4). Assuming a constant S phase duration, the nearly unchanged BrdU index indicates a stable cell division rate of the Langhans' cells, whereas the increased number of Ki67-positive Langhans' cells at term suggests a prolongation of the total cell cycle time and a diminished portion of resting cells. These data reveal the diminution of the Langhans' cell density within the trophoblast to be the most important factor in the reduction of trophoblast proliferation at term. However, there are some indications from studies on the differentiation of the Langhans' cells that not only proliferation, but also the rate of syncytial fusion, is changed during placental development and controls the regeneration of the syncytiotrophoblast (Kaufmann 1972; Arnholdt et al. 1988).

The results show, that trophoblastic proliferation is changed not only in the course of normal gestation, but also under adverse conditions such as in preeclampsia. In term placentae from preeclamptic women the data reveal a statistically significant elevation of the Ki67 index and a similar tendency for the BrdU index. Slightly elevated indices were reported by Krauer (1970b) and Hustin et al. (1984) using ^{3}H-thymidine autoradiography. In contrast, Kaltenbach et al. (1974) found no changes in the trophoblast proliferation rate. The only slight elevation in the labeling indices in these autoradiographic studies may be explained by the fact that the numbers of placentae and of the counted nuclei were small. Furthermore, most of the patients had only mild preeclampsia and were under treatment, as in our own study. In agreement with an elevated proliferation rate, there are morphometric data showing an increase in Langhans' cells in preeclampsia (Whigglesworth 1962; Fox 1964; Kaufmann et al. 1977; Jones and Fox 1980 and 1981; Soma et al. 1982; Teasdale 1985), even though such an increase could not be demonstrated in the present investigation (Fig. 6).

The greater variation in the Ki67/BrdU ratio in placentae from preeclamptic, compared with normal placentae (Table 2) and the remarkably low ratio in some of the preeclamptic placentae, indicate that changes in the cell cycle duration may be another factor in the regulation of the trophoblast proliferation under adverse conditions.

In contrast to the results in preeclampsia, evidence of an elevated proliferation rate in the placentae from women who smoked during pregnancy was not found in our study using Ki67 or BrdU, nor in an autoradiographic study by Toussaint (1989). Furthermore, we found a reduced number of Langhans' cells (Fig. 6), a result in accordance with a morphometric investigation by Teasdale and Ghislaine (1989).

The different results in preeclampsia and smoking are remarkable, because hypoxia has been suggested as the main pathogenetic factor in both conditions. In preeclampsia, placental ischemia is thought to be caused by a marked decrease in uteroplacental bloodflow, due in turn to an insufficent invasion of endovascular trophoblast into the myometrial segments of spiral arteries. The endothelium is not sufficiently replaced, and the elastic and muscular tissue of the arterial wall is not substituted by fibrinoid. Consequently, these vessels do not respond physiologically to vasomotor influences. With the onset of acute hypertension in preeclampsia, the vessels show characteristic arteriosclerotic changes, so-called atherosis (Dixon and Robertson 1961; Brosens and Renaer 1972; Robertson et al. 1975; Fox 1983; Soma et al. 1982). As a consequence, the placentae of preeclamptic women are characterized by infarcts, compensatory maturitas praecox, cytotrophoblastic hyperplasia and, at the ultrastructural level, focal syncytial necrosis and loss of syncytiotrophoblastic microvilli (Kaufmann et al. 1977; Jones and Fox 1980, 1981; Soma et al. 1982).

Placental ischemia is considered a major factor in the pathogenesis of the clinical effects of cigarette smoking during pregnancy (Naeye 1978; Philipp et al. 1984). Possible mechanisms by which smoking might exert its

effects are the nicotine-induced release of acetylcholine, epinephrine and nor-epinephrine causing vasoconstriction of uterine vessels and a reduction in perfusion of the intervillous space, or a reduction in the oxygen carrying capacity of the maternal blood by increased levels of carboxyhemoglobin (Pirani 1978). The expected increase in the proliferation rate was absent, as evidenced by both methods used in our study and by ^{3}H-thymidine-autoradiography in a study of Toussaint (1989). These results indicate that there may be a direct inhibitory effect on the proliferative and regeneratory activity of Langhans' cells. A reduced growth rate has also been shown in cell cultures of endothelial cells from smokers by Busacca et al. (1984) and by Karbowski et al. (1989).

In conclusion, our findings indicate that trophoblast proliferation is regulated by changes in the number of the Langhans' cells, not only in the course of normal gestation but also under pathological conditions. Another, probably more rapid mechanism for regulating the proliferation rate seems to be a change in the cell cycle duration. An increased syncytiotrophoblastic requirement for functional components such as cellular organelles or enzymes, which it cannot produce itself, could be the main triggering factor for these regulatory processes. Besides an accelerated maturation, increased trophoblastic regeneration is another mechanism contributing to the great compensatory capacity of the placenta. The results suggest that there are different pathogenetic mechanisms for placental impairment in preeclampsia and maternal smoking. In preeclampsia, injury to the syncytiotrophoblast, possibly of hypoxic origin, seems to provide a mechanism leading to an increased regeneration of the trophoblast resulting in a repair hyperplasia of the cytotrophoblast, as shown in morphometric studies. In opposition to this view, in placentae from mothers smoking during pregnancy there is a decreased number of Langhans' cells and no detectable change in the cell proliferation rate, probably following a direct toxic injury of the cytotrophoblast cells which are obviously unable to react by increased proliferation as in preeclampsia. No details are known about the regulation of trophoblast proliferation, either in the course of normal gestation or under pathological conditions. However, there are some data suggesting that growth factors may play an important role in this process (Arnholdt et al. 1990), and that the oncogene c-myc is expressed in actively developing tissue areas, but not in direct relation to the proliferation (Diebold et al. 1991).

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