Papers

Endothelial nitric oxide synthase immunoreactivity in early gestation and in trophoblastic disease

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Abstract

Aims—To study the localisation of the endothelial nitric oxide synthase (eNOS) in the normal placenta, with special emphasis on the implantation site in the first trimester of pregnancy, and in the different subtypes of trophoblastic cells in gestational trophoblastic disease.

Methods—The immunoperoxidase technique with an antibody directed against eNOS was applied to paraffin sections from first and second trimester placentas, placenta accreta, partial and complete hydatidiform moles, and choriocarcinoma. Immunoperoxidase staining for human placental lactogen (hPL) was performed on parallel sections.

Results—Prominent immunoreactivity for eNOS was found to be present in the intermediate trophoblastic cells of the cell columns of the anchoring villi and in trophoblastic cells at the implantation site. Staining was also present in the syncytiotrophoblast, most conspicuous at the apical cell border. In trophoblastic disease, proliferating large mononuclear cells, which were strongly positive for hPL, were found to be immunoreactive for eNOS.

Conclusions—eNOS immunoreactivity is strongly positive in the extravillous trophoblastic cells and to a lesser extent in the syncytiotrophoblast. In the former it may play a role in implantation and vascular invasion. Cells with differentiation to intermediate trophoblast in complete hydatidiform mole and choriocarcinoma also show high levels of eNOS, which may be associated with the haematogenous mode of spread of trophoblastic disease.

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Nitric oxide (NO) is a potent vasodilator, previously known as the endothelium derived relaxing factor (EDRF), produced and released by vascular endothelial cells.[1][2] It soon became apparent that NO has diverse biological functions, and is produced by cell types other than endothelium.[3][4]

The NO radical is generated by the action of the enzyme nitric oxide synthase (NOS). There are three distinct isoforms of this enzyme, two of which are constitutive calcium/calmodulin dependent (the endothelial and neuronal types), and the third is inducible and is not dependent upon calcium/calmodulin for its enzymatic action.[5][6] Of those, only the endothelial type of NOS is transcribed in the human placenta under normal conditions.[7][8]

Most of the research work on eNOS in the placenta has been concentrated on term placentas in normal and pathological conditions, especially those which may be the result of compromised blood supply to the fetus, for example preeclampsia.[8–12] The activity of eNOS in placental bed biopsies at term was found to be low, and somewhat higher at the basal plate.[11] Immunohistochemical studies, however, failed to show eNOS in the extravillous trophoblastic cells of the basal plate, neither was it identified at the implantation site from curettings of missed abortions (in contrast to terminations of pregnancy in our study).[13]

We studied the eNOS immunoreactivity on paraffin sections of placentas, especially from the first trimester of pregnancy, with emphasis on the implantation site. Intense immunohistochemical staining for eNOS was evident in the invasive trophoblastic cells—that is, the intermediate trophoblastic cells (also designated as extravillous trophoblastic cells or X cells). We further studied the localisation of eNOS in trophoblastic disease and found eNOS immunoreactivity in trophoblastic cells of the same—that is, intermediate—differentiation. We suggest that NO release by trophoblastic cells may play a role during the process of vascular invasion in placentation and in trophoblastic disease.

Methods

Paraffin blocks of formalin fixed tissues were selected from the files of the department of pathology at the Hadassah University Hospital, Mount Scopus. These included the following specimens:

1. Nine first trimester placentas (6–12 weeks' gestation by menstrual age).
2. Two early second trimester placentas (13 and 15 weeks' gestation).
3. Three uteruses with placenta accreta (from 30 weeks' gestation).
4. Four partial hydatidiform moles (diagnosed by morphological and cytogenetic analysis).
5. Four complete hydatidiform moles (diagnosed by morphological and cytogenetic analysis).
6. Four choriocarcinomas.

The paraffin blocks were sectioned at 5 µ and mounted on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). The slides were dried at 60°C for one hour and processed for immunohistochemical staining.

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A rabbit polyclonal antiserum, developed against a 20.4 kDa protein fragment corresponding to amino acids 1030–1209 of human eNOS, was used for immonohistochemical detection of eNOS on paraffin sections (Transduction Laboratories, Lexington, Kentucky, USA). Visualisation of eNOS in the sections was made by the streptavidin–biotin immunoperoxidase technique using Histostain-SP Kit (Zymed, San Francisco, California, USA) according to the manufacturer's directions.
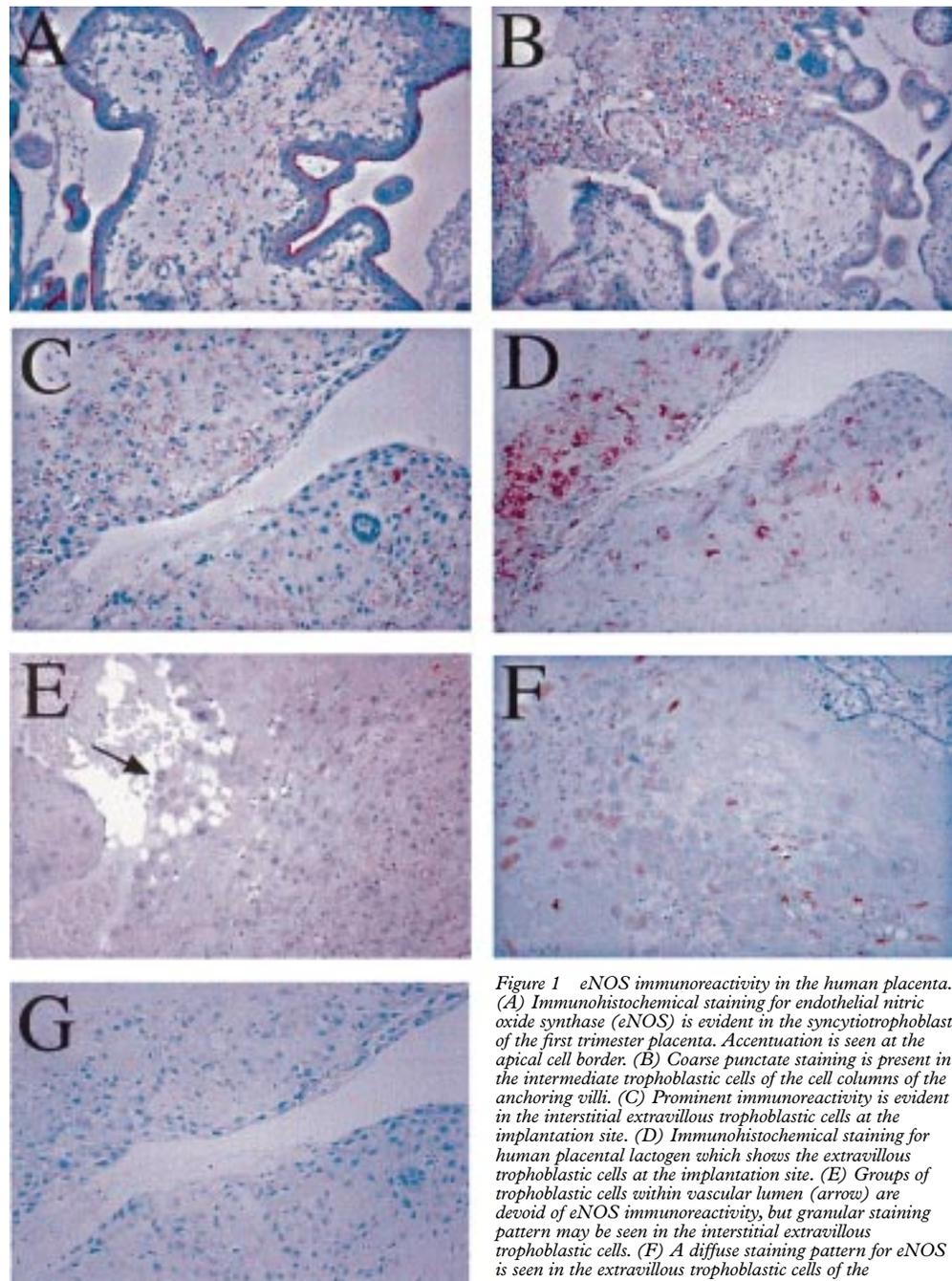

Figure 1   eNOS immunoreactivity in the human placenta. (A) Immunohistochemical staining for endothelial nitric oxide synthase (eNOS) is evident in the syncytiotrophoblast of the first trimester placenta. Accentuation is seen at the apical cell border. (B) Coarse punctate staining is present in the intermediate trophoblast cells of the cell columns of the anchoring villi. (C) Prominent immunoreactivity is evident in the interstitial extravillous trophoblastic cells at the implantation site. (D) Immunohistochemical staining for human placental lactogen which shows the extravillous trophoblastic cells at the implantation site. (E) Groups of trophoblastic cells within vascular lumen (arrow) are devoid of eNOS immunoreactivity, but granular staining pattern may be seen in the interstitial extravillous trophoblastic cells. (F) A diffuse staining pattern for eNOS is seen in the extravillous trophoblastic cells of the implantation site of placenta accreta. (G) Negative control without primary antibody (compare with (C)).

As a control the same procedure was performed in the absence of the primary antibody. At least one control was undertaken in each experiment, and at least one control was examined from each group of cases according to the diagnosis.

A rabbit antihuman placental lactogen (Zymed) was used to demonstrate the intermediate trophoblastic cells at the implantation site and in trophoblastic disease. The procedure was continued using the same Histostain-SP kit as above.

Results

In the first trimester placenta, weak diffuse immunohistochemical staining was present in the syncytiotrophoblast, with an increased intensity at the apical portion of the cell at the localisation of the microvillous brush border (fig 1A). Prominent coarse punctate staining was present in the intermediate trophoblasts of the cell columns of the anchoring villi (fig 1B).

Prominent positive immunoreactivity was also evident in the interstitial extravillous trophoblastic cells at the implantation site (fig 1C), which were identified as such by positive immunostaining for human placental lactogen (hPL) (fig 1D). In contrast to the interstitial intermediate trophoblastic cells, groups of trophoblastic cells detected within vascular lumens were devoid of eNOS immunoreactivity (fig 1E).

In the early second trimester placenta (weeks 13 to 15) the findings were similar. Later in pregnancy the staining for eNOS was present in the intermediate trophoblastic cells at the superficial implantation site of placenta accreta (fig 1F), but the staining pattern was rather diffuse and not punctate compared with placentas from earlier gestation. Immunoreactivity in the floating villi was very weak or undetectable.

In partial hydatidiform mole, diffuse staining with accentuation at the apical cell border was
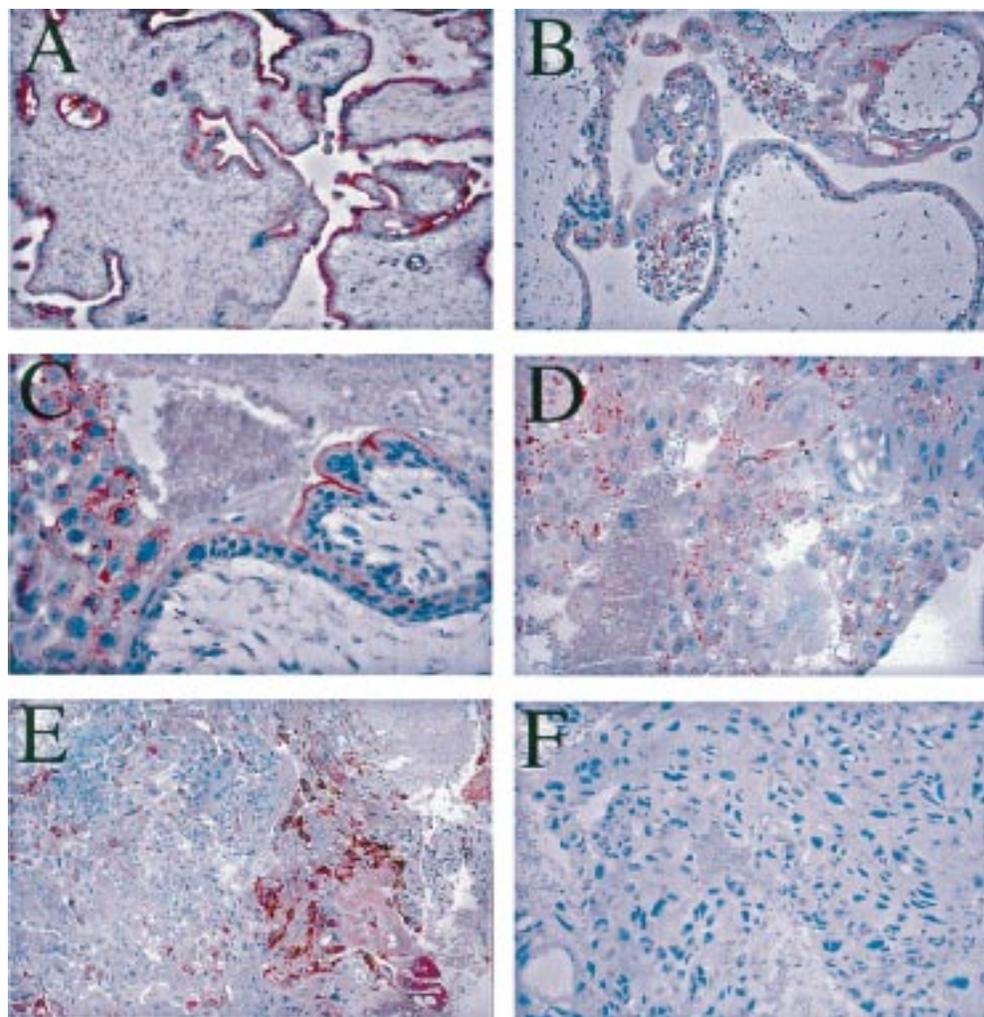

Figure 2   eNOS immunoreactivity in trophoblastic disease. (A) Partial hydatidiform mole: prominent immunoreactivity is evident in the syncytiotrophoblast with accentuation at the apical cell border. (B) Complete hydatidiform mole: endothelial nitric oxide synthase (eNOS) immunoreactivity is seen in the syncytiotrophoblast as well as in many of the proliferating mononuclear trophoblasts. (C) Higher magnification shows accentuation of the staining at the apical cell border of the syncytiotrophoblast and coarse granular staining pattern in the larger mononuclear trophoblastic cells, which correspond to intermediate trophoblastic cells. (D) eNOS immunoreactivity in choriocarcinoma parallels that of the normal placenta, with a diffuse staining pattern in syncytiotrophoblastic cells and a granular staining pattern in the larger trophoblastic cells with intermediate differentiation. (E) Choriocarcinoma: immunohistochemical staining for human placental lactogen demonstrating the mononuclear trophoblastic cells with intermediate differentiation. (F) Negative control without primary antibody.

present in the syncytiotrophoblast. The staining was generally more intense than in the normal first trimester placenta, and was also seen in trophoblastic inclusions typical of this entity (fig 2A). In complete hydatidiform mole, diffuse staining with increased intensity at the apical membrane was present in the villous bound and proliferating syncytiotrophoblast (fig 2, B and C). As in partial hydatidiform mole, the staining was generally more intense than in normal placenta, and sometimes prominent fine punctate staining was seen on the background of the diffuse cytoplasmic staining. In addition, prominent coarse punctate staining pattern was noted in the larger proliferating mononuclear trophoblastic cells, which were also positive for hPL, thus assuming intermediate trophoblastic differentiation. The same was found in choriocarcinoma—that is, diffuse, weak to moderate immunoreactivity of syncytiotrophoblastic cells and prominent punctate staining of the larger mononuclear trophoblastic cells (fig 2D), which were also positive for hPL (fig 2E).

No immunoreactivity was detected in any of the controls (figs 1G and 2F).

Discussion

The human placenta is a haemochorial placenta, which allows direct contact between the trophoblast and maternal blood. It is not surprising that the possible role of NO in this highly vascularised organ has been the subject of more than an occasional study. Research has concentrated mainly on eNOS in term placentas in normal and pathological conditions, especially those known to be associated with reduced uteroplacental blood flow, such as preeclampsia.[8–13] In the mature placenta eNOS was found to be expressed in the syncytiotrophoblast but not in the cytotrophoblast. This finding was confirmed by cell culture, in which NOS was expressed with differentiation of cytotrophoblastic cells and formation of syncytium.[13] We found that eNOS immunoreactivity was most prominent in the apical portion of the syncytiotrophoblast in the first trimester placenta, corresponding to the region with the most prominent histochemical reaction for NADPH diaphorase (indicating NOS activity), as was shown by Eis *et al*.[13] This localisation may be attributed to the antiadhesive–antiaggregating action of NO in a cell type which functions as endothelium in the fetomaternal circulation.[8 10 13 14] It should be mentioned here that eNOS differs from the other isoforms of NOS by having a membrane binding site at its N terminus, and is therefore in some instances predominantly membrane associated.[5]

Another site of intimate relationship between maternal blood and trophoblastic cells is the implantation site. At the early stages of gestation the placenta is a highly proliferative and invasive organ which penetrates the endometrium and maternal vessels in order to gain access to nutrients for the developing conceptus. The cell responsible for this invasive process is the extravillous trophoblastic cell,

also designated the intermediate trophoblast.[15] This type of trophoblastic cell resides in the cell columns and cell islands, basal plate, placental bed, and the chorion laeve. It has been suggested that the function of the interstitial extravillous trophoblastic cells is to prime the spiral arteries for their eventual invasion by trophoblastic cells.[16] There is evidence to suggest that this process is continued through the first trimester of pregnancy and a true fetomaternal circulation is achieved only by the end of the first trimester.[17–19] Relaxation of the vascular wall of vessels at the implantation site by NO produced by extravillous trophoblastic cells may play a role in this process.

In our present study we have shown prominent immunoreactivity for eNOS in the extravillous trophoblastic cells of the cell columns of the anchoring villi, in cell islands and at the basal plate in first trimester therapeutic abortions and in placenta accreta. This finding is in accord with appearance of eNOS in trophoblastic culture in vitro, together with differentiation and positivity for hPL.[13]

The marked reduction of eNOS immunoreactivity in trophoblastic cells within the lumen of decidual vessels, in contrast to the interstitial trophoblastic cells (fig 1G), is intriguing. It may be speculated that the interstitial extravillous trophoblastic cells are responsible for priming the decidual vessels for trophoblastic invasion, and once vascular conversion occurs the trophoblastic production of eNOS ceases, possibly with exposure to higher oxygen tension.

The prominent immunoreactivity for eNOS in the invasive cluster of trophoblastic cells prompted us to study eNOS in trophoblastic disease. We have shown that eNOS is produced by trophoblastic cells of partial and complete hydatidiform moles and in choriocarcinoma. The staining in molar disease was generally more intense than in the normal first trimester placenta. Moreover, it seems from our results that the most abundant eNOS immunoreactivity is present in the larger mononuclear trophoblastic cells and with the production of hPL—that is, with intermediate trophoblastic differentiation.

It has been shown in a chick chorioallantoic membrane model in vivo that eNOS plays an inhibitory role in the regulation of angiogenesis.[20] In this regard it is interesting to note that choriocarcinoma, in which we showed intense immunoreactivity for eNOS, does not develop an intrinsic blood supply and is dependent on vascular invasion of uterine vessels for oxygenation, similar to the events occurring in normal implantation.[21]

A few intriguing reports on the possible role of NO in neoplasia have been published recently. Marked reduction in NOS activity, as shown by histochemical methods, was found in colonic carcinomas and preneoplastic adenomas relative to non-neoplastic colonic mucosa.[22] A diverse pattern of nitric oxide gene expression, not always followed by nitric oxide generation, was found in human colon cancer cell lines.[23] In gynaecological cancer, activity and immunoreactivity for constitutive NOS

was found to be inversely related to tumour differentiation.[24] The inducible and constitutive isoforms of NOS were found to be expressed in breast cancer in cellular elements other than the epithelial tumour cells.[25 26] This expression was found to be correlated with tumour grade and metastatic potential. Further investigation is needed to elucidate the role of the different isoforms of NOS in neoplasia and metastasis.

Our findings in the present study, that expression of eNOS in trophoblastic disease is similar to its expression in the placenta, suggest that it may play a role in the vascular invasion of trophoblastic disease similar to its role in the process of implantation.

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