Cytotoxic effects of tumour necrosis factor (TNF)-α and interferon-γ on cultured human trophoblast are modulated by fibronectin

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Tumour necrosis factor (TNF)-α and interferon (IFN)-γ, produced by maternal inflammatory cells, may compromise trophoblast survival at the trophoblast–maternal interface and notably in the placental bed which is invaded by trophoblast. Extracellular matrix components, e.g. fibronectin, may enhance trophoblast survival. A possible protective effect of fibronectin against toxic effects of TNF-α and IFN-γ was investigated in cultured trophoblasts isolated from six human term placentas, grown on uncoated and fibronectin-coated plastics. IFN-γ and increasing doses of TNF-α resulted in decreasing viability of trophoblast on uncoated as well as fibronectin-coated dishes, as shown by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays, but for each TNF/IFN treatment condition viability on fibronectin was higher (P < 0.001). Epidermal growth factor (EGF), a growth factor reported to protect against TNF-α-induced toxicity, resulted in further increased viability, but not if IFN-γ was included in the treatment. EGF caused increased fibronectin secretion into the medium (P < 0.001), and double cytokeratin/fibronectin immunostaining confirmed the trophoblastic nature of fibronectin secreting cells. We conclude that fibronectin increases viability, but does not completely abolish the cytotoxic action of TNF-α and IFN-γ on trophoblast. The protective effect of EGF may be related to stimulation of fibronectin secretion by trophoblast.

Key words: epidermal growth factor/fibronectin/interferon-γ/trophoblast/tumour necrosis factor-α

Introduction

An intriguing aspect of reproductive biology is the survival and development of allogeneic fetal tissue within the potentially hostile environment of the uterus. In particular the trophoblast, representing the front line of uterine invasion, should be primarily vulnerable to maternal defence systems. In order to face this unique situation a complicated network of molecular signals between the trophoblast and the various cellular components of the uterus has evolved, and many of the cytokines peculiar to immune cell interactions play a role (Hunt, 1989; Guilbert et al., 1993). Aberrations in secretion patterns of these molecules could play a role in pregnancy complications such as pre-eclampsia (Conrad and Benyo, 1997), a condition characterized by abnormal trophoblast invasion into the uterine wall (Brosens et al., 1972; Pijnenborg, 1994).

One of the molecules thought to be involved in these interactions is tumour necrosis factor (TNF)-α. This compound forms part of the normal Th1 response in foreign tissue rejection, which would normally be overcome by the predominant Th2 response characteristic of the pregnant condition (Wegmann et al., 1993). Increased serum concentrations of this cytokine in pre-eclamptic patients may indicate a disturbance of the Th1:Th2 balance and is possibly related to the inhibition of trophoblast invasion (Keith et al., 1995). An immunolocalization study of TNF-α provided evidence of increased expression of this cytokine in the placental bed of pre-eclamptic patients (Pijnenborg et al., 1998). Direct evidence for a cytotoxic effect of TNF-α has been provided by in-vitro experiments on isolated human trophoblasts, illustrating viability loss and apoptosis after TNF-α treatment, an effect which is enhanced by interferon (IFN)-γ (Yui et al., 1994).

In the latter, as in most other in-vitro studies, trophoblasts were encouraged to syncytialize by plating the isolated cytotrophoblastic cells at high densities. In vivo, however, different types of trophoblast are involved in placentation, and it can be expected that distinct cell types may react differently to various stimuli. Our in-vitro approach consists of plating the cells at low densities and, as a result, the majority of cells remain cytотrophoblastic, and mimic some of the characteristics normally shown by invasive extravillous trophoblast, e.g. an extensive immunoreactivity for human prolactin (Pijnenborg et al., 1996). Furthermore, in this previous study we were impressed by the improved viability of the trophoblast plated on fibronectin-coated dishes, compared with cultures on uncoated plastics. Since extracellular matrix components were not used in previously reported experiments (Yui et al., 1994), we decided to investigate the possible interference of fibronectin with TNF-α induced cytotoxic effects on trophoblast. Since epidermal growth factor (EGF) had previously been shown to protect the trophoblast against the toxic effects of TNF-α and IFN treatment (García-Lloret et al., 1996), this factor was included in our experiments. Finally, the intrinsic capacity of
the trophoblast to secrete fibronectin in response to cytokine supplementations was investigated, since this may indicate the existence of a mechanism by which the cells can protect themselves against adverse conditions.

Materials and methods

Cell culture
Trophoblastic cells were isolated from six term placentae of uncomplicated pregnancies. Mean gestational age (± SD) was 39.6 ± 1.2 weeks, mean birth weight was 3307.5 ± 325.5 g. The purification procedure was based on the Klinman method (Klinman et al., 1986), modified by preliminary overnight incubation of the minced placental tissue in 0.25% trypsin solution (Life Technologies, Merelbeke, Belgium) at 4°C (Pijnenborg et al., 1996), followed by two centrifugation sequences over discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden). As a final purification step, the cell suspension resulting from Percoll gradient centrifugation was subsequently purified by removal of CD45-positive contaminating cells using antibody-coated Dynabeads® M-450 (Dynal, Oslo, Norway) after 20 min incubation at 4°C followed by magnetic separation. The purified cytотrophoblastic cells were resuspended in culture medium (Dulbecco’s modified Eagle’s medium low D-glucose, Life Technologies) with 20% fetal calf serum (FCS), and plated at a concentration of 200,000 cells/ml into 96-well plates as well as in eight-chambered LabTek slides (Nunc, VEL, Leuven, Belgium). Both multi-well plates and LabTek slides were either precoated with 1 h incubation with 0.1 mg/ml human plasma fibronectin (Life Technologies), or left uncoated. The plates and slides were cultured at 37°C in 5% CO₂ in air.

Cytokine treatment
After 24 h of culture, the supernatants were removed, the slides were washed and fresh medium with or without FCS was added. In half the cultures the fresh serum-free medium was supplemented with 5 ng/ml EGF (Sigma Chemical Co) by functional mitochondria to formazan. After a 2 h incubation at 37°C of the cells with MTT, the cells were lysed with dimethyl sulfoxide in Sorenson’s glycine buffer and the formazan crystals solubilized. Absorbance was read at 550 nm using a spectrophotometric microplate reader.

MTT assay
As a measure of viability the MTT assay was performed on cells cultured in multi-well plates (Mosman, 1983; Keith et al., 1995). The assay depends on the reduction of the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co) by functional mitochondria to formazan. After a 2 h incubation at 37°C of the cells with MTT, the cells were lysed with dimethyl sulfoxide in Sorenson’s glycine buffer and the formazan crystals solubilized. Absorbance was read at 550 nm using a spectrophotometric microplate reader.

Immunochemistry
Immunostaining for cytokeratin and vimentin was performed as a quality control for trophoblast cell cultures. To facilitate evaluation, a double-staining procedure was developed. After destroying endogenous alkaline phosphatase activity by incubation in HCl (0.2N, 10 min) mouse anti-vimentin monoclonal antibody (clone V9, Dako, Glostrup, Denmark; diluted 1/300) was detected in a first staining sequence with goat anti-mouse antibodies (Dako; 1/50) followed by mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (Dako; 1/100). In a second sequence fluorescein isothiocyanate (FITC)-conjugated mouse anti-cytokeratin monoclonal antibody (clone CAM 5.2, Becton Dickinson, Erembodegen, Belgium; diluted 1/400) was detected with rabbit anti-FITC antibodies (Dako; 1/4000) followed by peroxidase-conjugated goat anti-rabbit antibodies (1/100; Jackson Immunoresearch Laboratories, West Grove, PA, USA). To prevent cross-reactivity between both immunohistochemical sequences the slides were incubated with normal mouse serum (Dako; 1/10) after the first sequence. All antibodies were diluted in Tris-buffered saline (TBS), and were applied for 30 min at room temperature. Before applying antibody dilutions, non-specific protein binding was blocked by preincubation with normal goat serum (Dako; 1/30), with addition of Tween-80 (Merck; 0.5%). Complement inactivated normal human serum (1/25) was added to all secondary antibody dilutions. For chromogens, we used naphthol-ASMX-phosphate/Fast Blue BB (Sigma) and aminoethylcarbazole (Sigma) respectively, resulting in blue staining of vimentin-positive contaminating cells and red staining of cytokeratin-positive trophoblast. Cells were counterstained with Methyl Green (1% in sodium citrate 0.02 mol/l; 45 min). Only cultures showing at least 85% cytokeratin-immunostained trophoblast were further included in the study.

The absence of cross-reaction between first and second staining sequence was shown by substituting buffer for the FITC-conjugated anti-cytokeratin monoclonal. The accuracy of the double staining methodology was further controlled by comparing cell counts for both antibodies obtained by double and single staining protocols.

Double immunostaining for cytokeratin and fibronectin was also performed in two sequences. In the first sequence goat anti-human fibronectin polyclonal antibodies, reacting with all fibronectin types (AES 504; Sera-lab; 1/300), were detected with peroxidase-conjugated rabbit anti-goat antibodies (Dako; 1/80) and aminoethylcarbazole as chromogen, while in the second sequence mouse anti-cytokeratin monoclonal antibodies (clone CAM 5.2 un conjugated; 1/30) were detected with goat anti-mouse antibodies followed by mouse APAAP complex and Fast Blue BB as chromogen. Additional procedure-conditions were the same as for anti-vimentin/anti-cytokeratin staining, except that cross-reaction between both sequences was prevented by incubation with normal goat serum (Dako; 1/10), and protein blocking was omitted before applying anti-fibronectin. The procedure resulted in blue staining of trophoblast, with superimposed red staining if fibronectin was present.

Control experiments where the anti-vimentin and anti-cytokeratin antibodies were substituted by either buffer or irrelevant immunoglobulins of the same subclass, or where only the first sequence (for anti-fibronectin) was carried out substituting the anti-fibronectin by the anti-cytokeratin antibody resulted in negative staining for the substituted antibody.

TUNEL staining
A modification of the TUNEL procedure (Gavrieli et al., 1992) was used to detect apoptosis. Briefly, cells were post-fixed in freshly prepared paraformaldehyde buffered with phosphate-buffered saline (PBS) (4%; 30 min at 4°C), rinsed, permeabilized in Triton X-100 (Sigma T8787, molecular biology grade; 0.2%; 15 min) and incubated (1 h at 37°C) in the TUNEL reaction mixture, containing terminal transferase (150 IU/ml), digoxigenin-11-dUTP (2.5 nmol/ml), dATP
reaction was stopped with a 2.5 mol/l H2SO4 solution, and absorbance diaminedihydrochloride (Sigma) was added, after 15 min the for 1 h at room temperature. Subsequently the substrate peroxidase conjugate; Life Technologies) was added and incubated washing, secondary antibody (goat anti-rabbit immunoglobulins – were added to the coated plates and incubated overnight at 4 °C. Antibody (1/20 000 rabbit polyclonal antiserum; Life Technologies) resulted in a stepwise decreased viability 24 h after treatment, although the differences within the groups were not signi fi cant. However, for each of the TNF-α ± IFN-γ treatment conditions, viability of cells cultured on fibronectin was significantly higher than those cultured on uncoated plastics (P < 0.001, Figure 1A). Four days after TNF-treatment, the stepwise decline in viability was still apparent in all conditions, but the decreased viability after IFN-γ addition versus non-treated cells was now significant in cultures on fibronectin (P < 0.05 for IFN-γ only; P < 0.001 for both 100 and 1000 IU TNF-α with IFN-γ). In conditions with IFN-γ treatment, cultures on fibronectin were no longer significantly more viable than corresponding cultures on uncoated plastics, while conditions with TNF-α but without IFN-γ kept the same level of significance compared with uncoated recipients (P < 0.001, Figure 1B) as seen 24 h after treatment.

**Fibronectin assay**

Culture media, recovered from LabTek chamber slides, were assayed for the presence of soluble fibronectin using an enzyme-linked immunosorbent assay (ELISA) (Hoeben et al., 1995). Briefly, multiwell plates were coated overnight at 4°C with fibronectin (human plasma fibronectin; Life Technologies). After washing and blocking of the non-coated sites, preincubated (overnight at 4°C) mixtures of the culture medium samples and anti-human fibronectin antibody (1/20 000 rabbit polyclonal antiserum; Life Technologies) were added to the coated plates and incubated overnight at 4°C. After washing, secondary antibody (goat anti-rabbit immunoglobulins–peroxidase conjugate; Life Technologies) was added and incubated for 1 h at room temperature. Subsequently the substrate o-phenylenediaminedihydrochloride (Sigma) was added, after 15 min the reaction was stopped with a 2.5 mol/l H2SO4 solution, and absorbance at 492 nm was read using a spectrophotometric plate reader.

**Statistical analysis**

Data from the MTT, fibronectin ELISA and TUNEL assays were analysed using analysis of variance (ANOVA), with application of Tukey’s post-hoc test if P < 0.05.

**Results**

**Viability of TNF-α/IFN-γ treated trophoblast cultured on uncoated versus fibronectin-coated plastics**

MTT tests showed that increasing doses of TNF-α and IFN-γ resulted in a stepwise decreased viability 24 h after treatment, on uncoated as well as on fibronectin-coated recipients, although the differences within the groups were not significant. However, for each of the TNF-α ± IFN-γ treatment conditions, viability of cells cultured on fibronectin was significantly higher than those cultured on uncoated plastics (P < 0.001, Figure 1A). Four days after TNF-treatment, the stepwise decline in viability was still apparent in all conditions, but the decreased viability after IFN-γ addition versus non-treated cells was now significant in cultures on fibronectin (P < 0.05 for IFN-γ only; P < 0.001 for both 100 and 1000 IU TNF-α with IFN-γ). In conditions with IFN-γ treatment, cultures on fibronectin were no longer significantly more viable than
**Effect of EGF on viability of trophoblast after TNF-α treatment, on uncoated and fibronectin-coated plastics**

By 24 h after TNF ± IFN-γ treatment of cells cultured on uncoated plastics, no significant increase in viability was provided by EGF supplementation in each treatment condition, although the stepwise decrease in viability caused by TNF-α and IFN was no longer apparent (Figure 1A). In cells cultured on fibronectin, the (non-significant) stepwise decrease in viability was not prevented by EGF, except for a slightly improved effect in cultures treated with 100 IU TNF-α only (P < 0.05). When we compare the four conditions of plastic/fibronectin without or with EGF supplementation, we conclude that culture on fibronectin without EGF does not lead to a significantly increased viability versus cultures on plastic with EGF, except for cells not treated with TNF-α or IFN-γ (P < 0.01; significance levels not indicated on Figure 1); cells on fibronectin with the addition of EGF are significantly more viable than cells on plastic without (P < 0.001) and with (P < 0.001) EGF for each of the TNF/IFN treatment conditions (Figure 1A). By 4 days after TNF-treatment, the same tendency was noted as after 24 h: no significantly increased viability with addition of EGF in cells cultured on plastics, while the stepwise decrease with TNF/IFN treatment on plastic was again apparent; EGF supplementation did not result in a significantly increased viability in cells cultured on fibronectin, except for a slightly positive effect in 100 and 1000 IU TNF-α treated cells without IFN (P < 0.05, Figure 1B). Comparison of the four fibronectin/plastic ± EGF conditions led to similar results as after 24 h: culture on fibronectin without EGF does not result in significantly increased viability versus cultures on plastic with EGF, except for cells not treated with TNF-α or IFN (P < 0.01); cells on fibronectin with EGF were significantly more viable than cells cultured on plastic without and with EGF, but this time only for treatments without IFN (P < 0.001, Figure 1B).

**Evaluation of apoptosis**

TUNEL staining for evaluation of apoptotic nuclei was performed on LabTek slides from the extreme treatment conditions only, i.e. no treatment versus 1000 IU TNFα + IFN-γ. Mean percentages of labelled nuclei were always higher at 4 days than at 1 day post-treatment. Results of nuclear counting were variable however, and no significant difference was found between different culture conditions both at 24 h and 4 days after treatment (Table I).

**Fibronectin production of cultured trophoblastic cells**

Fibronectin ELISAs were performed on culture media derived from cultures on uncoated plastics only, in order to avoid any interference of the assay with fibronectin-precoating. After 24 h of TNF-α/IFN-γ treatment, values found in EGF supplemented cultures were consistently higher, but although analysis of variance (ANOVA) showed a significance level of P < 0.05, no individual significant differences could be discerned. Higher fibronectin concentrations after EGF treatment were more pronounced 4 days after treatment (Table II), and ANOVA showed a significance level of P < 0.001. In this case addition of EGF to 1000 IU TNF-α treated cultures showed a significantly increased soluble fibronectin release (P < 0.01), but addition of IFN-γ resulted in significantly lower fibronectin secretion in this condition (P < 0.05; Table II).

In order to confirm that the soluble fibronectin was secreted by the trophoblast cells in culture, and not by the occasional contaminating cells, double immunostaining for cytokeratin and fibronectin was performed. This double staining was applied to non-treated cells cultured on plastic only. Mixed colour staining (cytokeratin blue, fibronectin red) was obtained in the majority of the cultured cells (92 ± 4.4% fibronectin containing cytokeratin-positive cells), confirming the trophoblastic nature of the fibronectin secreting cells (Figure 2).

**Discussion**

The existence of a cytokine network determines selective survival, growth and differentiation of different cells at the maternal–fetal interface and in particular in the placental bed, where extravillous trophoblast invades the uterine wall. For unravelling the interactions of the various cell types various approaches are needed, and especially isolation of particular cell types to study their behaviour in vitro, alone or in combination, offers an attractive research strategy. Unfortunately, it is very difficult to set up a reliable culture system of extravillous trophoblast, and out of necessity one often has to use villous cytotrophoblastic cells isolated from delivered placentae. Nevertheless, villous cytotrophoblastic cells in vitro, liberated from their normal tissue surroundings, may develop characteristics of a more general trophoblastic cell phenotype, which may provide at least an indication of processes taking place in the placental bed in vivo.

Macrophages play an important role in regulating placentation (Hunt, 1989; Vince and Johnson, 1996). A typical cytokine product of activated macrophages is TNF-α. This cytokine, alone or in combination with IFN-γ, is cytotoxic for cultured villous trophoblasts (Yui et al., 1994). In the latter experiments cells were plated at high density (1 × 10⁶ cells/ml) to encourage syncytialization, thus producing a culture system representative for (villus) syncytiotrophoblast. Following TNF-α treatment, whole sheets of syncytium were detached and washed off during further processing, resulting in a significant drop in MTT absorbance. In the presently reported experiments, we aimed at maintaining a mainly mononuclear (cytotrophoblastic) culture (Pijnenborg et al., 1996) by plating the cells at low (200 000 cells/ml) density. We did not notice detachment of cells under microscopic examination, but TNF-α and IFN-γ treatment repeatedly showed a decreased (but non-significant) MTT absorbance (Figure 1). The more subtle results reported here are also reflected by variable and non-significant results of TUNEL-staining to reveal apoptosis. In contrast to previous data (Yui et al., 1994), IFN-γ alone was more toxic than a high dose (1000 IU) TNF-α alone, which may indicate a different sensitivity of our culture system. TNF-α may have a range of responses (apoptosis, cell proliferation, differentiation) which are mediated by differential expression of the two receptors together with their associated signal transduction.
Fibronectin and TNF-α/IFN-γ effects on trophoblast

Table I. Percentage of apoptotic trophoblastic cells$^a$ after treatment with tumour necrosis factor (TNF)-α and interferon (IFN)-γ in the presence or absence of epidermal growth factor (EGF) ($n = 6$)

<table>
<thead>
<tr>
<th></th>
<th>24 h</th>
<th>4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TNF/IFN</td>
</tr>
<tr>
<td>PL</td>
<td>6 ± 3.4</td>
<td>8 ± 4.1</td>
</tr>
<tr>
<td>FN</td>
<td>6 ± 2.3</td>
<td>6.5 ± 2.4</td>
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</table>

$^a$Percentage of TUNEL-positive nuclei (mean ± SD) in cells cultured on uncoated (PL) or fibronectin-coated (FN) plastics. Untreated cultures were compared with high cytotoxic dose (1000 IU TNF-α + 100 IU IFN-γ/ml) treated cultures only.

Table II. Fibronectin concentration (ng/ml) in supernatants of trophoblast cultured on uncoated plastic, in absence and presence of epidermal growth factor (EGF) ($n = 6$)$^a$

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>100 TNF</th>
<th>1000 TNF</th>
<th>IFN</th>
<th>IFN + 100 TNF</th>
<th>IFN + 1000 TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>− EGF</td>
<td>31.6 ± 26.2</td>
<td>30.6 ± 29.4</td>
<td>25.1 ± 16.6</td>
<td>38.2 ± 35.9</td>
<td>25.2 ± 14.5</td>
</tr>
<tr>
<td></td>
<td>+ EGF</td>
<td>40.7 ± 22.3</td>
<td>40.0 ± 20.9</td>
<td>39.6 ± 16.4</td>
<td>39.4 ± 13.7</td>
<td>39.4 ± 16.9</td>
</tr>
<tr>
<td>4 days</td>
<td>− EGF</td>
<td>57.9 ± 30.3</td>
<td>52.9 ± 23.9</td>
<td>68.9 ± 27.5</td>
<td>101.2 ± 49.3</td>
<td>58.9 ± 27.3</td>
</tr>
<tr>
<td></td>
<td>+ EGF</td>
<td>250.5 ± 146.7</td>
<td>252.4 ± 147.3</td>
<td>355.7 ± 333.4$^b$</td>
<td>146.9 ± 85.8</td>
<td>118.9 ± 60.7</td>
</tr>
</tbody>
</table>

$^a$ Cultures after 24 h showed a significant difference ($P < 0.05$) using analysis of variance (ANOVA), but no individual significant differences were found. After 4 days' culture ANOVA showed $P < 0.001$; $^b P < 0.01$ versus the same 1000 IU tumour necrosis factor (TNF)-α treatment without EGF; $^c P < 0.05$ versus 1000 IU TNF-α treatment with EGF without interferon (IFN).

Figure 2. Trophoblastic cells, cultured on uncoated plastics, double-stained for cytokeratin (blue) and fibronectin (red). Scale bar = 17.5 μm.

pathways (Liu et al., 1996; Natoli et al., 1997). It has been shown that TNF-α receptors are expressed differentially with respect to gestational age by human placental cells and fetal membranes (Yelavarthi and Hunt, 1993). It is not unlikely that isolated trophoblastic cells may undergo various shifts in receptor expression and/or transduction pathways in different culture conditions, which may explain the differences in apoptotic response as found by Yui et al. (1994) and the present studies.

We considered that adding an extracellular matrix component would provide a further step to a more ‘physiological’ culture condition, thus approaching reality more closely. Culturing trophoblast on fibronectin-coated substrates facilitates their flattening and spreading (Kao et al., 1988; Burrows et al., 1993). Since trophoblast grown on fibronectin shows an aesthetically more pleasing morphology, we considered that this condition might enhance viability, and we therefore wondered whether fibronectin coating could offer protection to toxic effects of TNF-α and/or IFN-γ. The present experiments showed a definite increase of trophoblast viability when cultured on fibronectin, but adding TNF-α without or with IFN-γ still showed a stepwise deterioration, which was significant in the presence of IFN-γ after 4 days of culture. Cells on fibronectin treated with TNF-α alone were always more viable than cells on uncoated plastic under equivalent cytokine treatment. Therefore, fibronectin offers protection to TNF-α-induced toxicity, but not in the presence of IFN.

Addition of EGF has been shown to offer protection to TNF-α/INF-γ toxicity on syncytial trophoblastic cells cultured on plastic (Garcia-Lloret et al., 1996). In our experiments, we also included EGF exposure for cultures on plastic as well as on fibronectin. By 24 h after TNF-α without or with IFN treatment, the normal stepwise decrease in viability did not occur in EGF treated cultures on plastic, but this was no longer the case after 4 days of culture. In all conditions tested, EGF pretreatment of trophoblast on fibronectin definitely provided the best viability results, but the toxic effects of IFN were again apparent after 4 days culture (Figure 1B). It is possible that our EGF dose could be suboptimal and therefore unable to overcome the toxic effects of IFN-γ, but no additional experiments were performed for further elucidation of this point. Since both addition of EGF and culturing on fibronectin showed beneficial effects, we examined the possible link between the two by investigating whether EGF did stimulate fibronectin production by the cultured cells. Our ELISA results...
on the culture supernatants provide evidence for such a stimulatory effect. Furthermore, double immunostaining on fixed cultured cells confirmed that the fibronectin was produced by the trophoblastic cells and cannot be considered merely as a byproduct released by the occasional contaminating fibroblasts. Whether or not the fibronectin released in the culture medium was of a trophoblast-specific subtype could not be determined by the assay used.

In the uterine wall during pregnancy EGF is produced by decidual stromal and glandular epithelial cells (Hofmann et al., 1991), and the trophoblast, besides producing the factor itself, also possesses receptors for EGF (Mühlhauser et al., 1993; Duello et al., 1994). Secretion of extracellular matrix proteins certainly belongs to the repertoire of cell behaviour that stabilizes their position within the tissue, and therefore enhances their survival. Other growth factors may have similar effects to EGF. For example, TGF-α is known to stimulate oncofetal fibronectin synthesis by trophoblast (Feinberg et al., 1994), and also M-CSF, which is secreted by uterine epithelial and glandular epithelial cells, has been shown to stimulate fibronectin production by trophoblast (Omigbodun et al., 1998).

On the other hand, TNF-α inhibits production of oncofetal fibronectin by first trimester trophoblast in vitro (Meisser et al., 1999), while our results on term trophoblast indicated lower fibronectin secretion after TNF-α + IFN-γ compared with TNF-α only. In-vivo trophoblastic cells surround themselves with fibronectin; this is illustrated by immunohistochemical studies in young implantation sites (Earl et al., 1990) as well as term placental bed specimens (Pijnenborg et al., 1992). In the latter study, it was apparent that pericellular fibronectin deposition occurs mainly in interstitially invading trophoblast, and is not present in the spiral artery walls where trophoblast is embedded in a fibrinoid matrix, although young stages are reported to show fibronectin immunostaining in their invasive endovascular cells (Earl et al., 1990). Of course it remains questionable as to how far the in-vivo experiments reflect the reality of the placental bed.

The present observations may have some relevance to the still elusive pathogenesis of pre-eclampsia. The earliest defect occurring in this condition is a restriction of endovascular trophoblast invasion and associated conversion of the uterine spiral arteries (Brosens et al., 1972). It is not known whether or not this condition is associated with an actual killing or destruction of invading trophoblast by an over-reactive maternal inflammatory response. Non-invaded spiral arteries may undergo atherotic changes, a process accompanied by infiltration of macrophages which are transformed into lipophages. The occasional occurrence of trophoblastic cell remnants have been reported (McFadyen et al., 1986; Meekins et al., 1994), which may suggest that invading trophoblast were killed before or during the atherogenic process. The extent and mechanism of this killing needs further investigation, but it is tempting to suggest a role for TNF-α in the process. Indeed the serum concentrations for this cytokine are elevated in pre-eclamptic women (Keith et al., 1995) and TNF-α could be localized within the macrophages of atherotic spiral arteries (Pijnenborg et al., 1998). Pre-eclampsia is also associated with elevated plasma concentrations of fibronectin (Lazarchick et al., 1986; Ballegeer et al., 1989). The in-vivo observations of increased concentrations of both serum TNF-α and plasma fibronectin in pre-eclampsia may at first sight look paradoxical, but blood concentrations do not necessarily reflect the cellular processes taking place in the placental bed. The increased plasma fibronectin in pre-eclampsia is of the cellular type (Lockwood and Peters, 1990), and is thought to result from endothelial disruption which forms the basis for the second stage of the disease (Roberts et al., 1989). Whether placental and placental bed trophoblast contributes directly to this increased plasma fibronectin in the presence of increased TNF-α is unlikely following the data of Meisser (Meisser et al., 1999) and the present data.

In conclusion, our in-vitro data suggest a mild protective role for fibronectin to the cytotoxic action of TNF-α, but not when IFN is included. EGF induces fibronectin secretion by cultured trophoblast and may in this way contribute to the protective effect exerted by the extracellular matrix. In our low-density culture system, which precludes the formation of extensive syncyial masses, cytotoxic effects of the added cytokines are not, however, so dramatic as shown by the non-significant changes on apoptosis. It is tempting to relate this to the mainly mononuclear trophoblastic cell type that is maintained in our culture conditions, and which is different from other published work. This may once more illustrate the difficulty of relating in-vitro findings on isolated trophoblast to the complex reality of the maternal–fetal interface in vivo.

References


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