The role of transforming growth factor-α (TGF-α)-epidermal growth factor receptor (EGFR) interactions in regulating benign and malignant trophoblast proliferation were examined. Benign cytotrophoblast (CT) demonstrated mitogenic stimulation in response to TGF-α; BeWo and JAr choriocarcinoma cell lines failed to respond. EGFR levels in BeWo and JAr were determined by enzyme linked immunoassay (ELISA) to be at least 10-fold higher than those in benign CT. EGFR isolated from BeWo and JAr also demonstrated functional tyrosine kinase activity. Using a combination of immunoperoxidase (IP) and ELISA techniques, choriocarcinoma cells were found to produce significant quantities of TGF-α that were comparable with those reported previously by this laboratory for benign CT, and were felt to be stimulating their own proliferation in an autocrine fashion. EGFR blocking and TGF-α neutralizing antibodies inhibited JAr proliferation whereas an EGF neutralizing antibody did not. The data presented here and in our previous report indicate that a TGF-α–EGFR autocrine loop may regulate normal and malignant CT proliferation. Choriocarcinoma cells may be proliferating at a maximal rate due, in part, to EGFR overexpression and are therefore unable to respond further to exogenous growth factor. Thus, EGFR overexpression may contribute to the uncontrolled proliferation of choriocarcinoma cells in general.

© 1997 W. B. Saunders Company Ltd

INTRODUCTION

Placental chorionic villi, where waste and nutrients are exchanged between the maternal and fetal circulatory systems, are covered by two layers of trophoblastic cells. The outer multinucleated syncytiotrophoblasts (ST) are terminally differentiated and are the primary source of hormones such as human chorionic gonadotropin (hCG). The inner cytotrophoblasts (CT) are undifferentiated mononuclear cells and are the source of both the ST and extravillous CT. The latter are highly proliferative cells responsible for much of the rapid growth and invasive behaviour of the early placenta. This 'pseudomalignant' behaviour is tightly regulated and CT proliferation and invasion cease to a large extent after midgestation. Continued proliferation may occur in a benign neoplastic lesion known as the hydatidiform mole. Uncontrolled proliferation, invasion of maternal tissues and metastasis occur in the fully malignant chorionic carcinoma (reviewed in Ringler and Strauss III, 1990; Aplin, 1991). Because it exhibits a continuum of phenotypes ranging from pseudomalignant to fully malignant, the placenta provides a useful model system in which to study normal cell behaviour and tumorigenesis.

Numerous growth factors appear to play a role in controlling growth and differentiation in normal and neoplastic placenta. Evidence suggests that interactions between the epidermal growth factor receptor (EGFR) and transforming growth factor-α (TGF-α), one of several EGFR ligands, may influence placental development. We have previously demonstrated EGFR in both CT and ST throughout gestation (Filla, Zhang and Kaul, 1993); similar results have been reported by others (Tavare and Holmes, 1989; Bulmer, Thrower and Wells, 1989; Kawagoe et al., 1990). EGFR have also been demonstrated in molar trophoblasts (Carson et al., 1983; Ladines-Llave et al., 1993) and choriocarcinoma cells by a variety of methods (Miyachi et al., 1990; Ilekis and Benveniste, 1985; Chen et al., 1990; Ladines-Llave et al., 1993). TGF-α has been localized to human endometrium, decidua and trophoblast (Haining et al., 1991; Filla, Zhang and Kaul, 1993; Hofmann et al., 1993; Horowitz et al., 1993; Lysiak, Han and Lala, 1993), and TGF-α mRNA and/or protein have been isolated from whole placentae throughout gestation. Protein levels have been reported to be several orders of magnitude higher than EGF (Stromberg et al., 1982; Bissonnette et al., 1992). Our laboratory has identified significant quantities of TGF-α in serum-free (SF) culture media conditioned by human CT (Filla, Zhang and Kaul, 1993), and we, along with others, have demonstrated that TGF-α is mitogenic for human and mouse trophoblasts (Filla, Zhang and Kaul, 1993; Iguchi et al., 1993; Haimovici and Anderson, 1993; Lysiak, Han and Lala, 1993).
Relatively little has been reported regarding the effects of TGF-α on molar trophoblasts or choriocarcinoma cells. The purpose of this study was to define further the roles of TGF-α-EGFR interactions in regulating normal and neoplastic placental development, and to determine whether changes in either contribute to the hyperproliferative behaviour of choriocarcinoma cells.

**MATERIALS AND METHODS**

**Tissue procurement and cell culture**

Fresh placental tissues from elective abortions [8–16 weeks menstrual age (MA)] were received immediately from surgery into surgical pathology and were processed for cell culture. Culture of benign CT was performed in a manner similar to that described by Goustit et al. (1985). Cells were cultured in standard media consisting of F12/DME, 10 per cent fetal bovine serum (FBS), 0.1 per cent glucose and 1 per cent antibiotics (penicillin, 100 U/ml; streptomycin, 100 mg/ml) in Primaria flasks (Falcon, Lincoln Park, NJ, USA) coated with 1 μg/cm² fibronectin (Sigma, St. Louis, MO, USA). In order to determine purity of the isolated cultures various marker antigens were examined in cells grown on glass coverslips and fixed according to Laurila et al. (1978). The antigens that were screened for included placental alkaline phosphatase (PAP), hCG, human placental lactogen (hPL), vimentin, keratin, Mac 387 and factor VIII. PAP, hCG, hP.I and factor VIII were stained using antibodies described previously (Filla, Zhang and Kaul, 1993). Mac 387 was purchased from Dako (Carpentaria, CA, USA). Vimentin was stained with polyclonal anti-vimentin 68-120-1 (ICN, Costa Mesa, CA, USA) and keratin was stained with polyclonal anti-keratin 260 raised against type I and II keratins (courtesy of Jonathan Jones, Department of Cell, Molecular and Structural Biology, Northwestern University Medical School, Chicago, IL, USA). IP studies were performed in conjunction with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) using the protocol supplied by the manufacturer, and demonstrated greater than 95 per cent of cells staining positive for keratin; focal positive staining for hPL was also observed consistent with CT data (data not shown); other markers were negative. We did not identify these cells as being derived specifically from villous or extravillous CT populations.

BeWo and JAr choriocarcinoma cell lines were obtained from ATCC and were cultured in either F12/DME, 10 per cent FBS, 0.1 per cent glucose and 1 per cent antibiotics or F12/DME, 2 per cent TCH defined media supplement (Celox, Hopkins, MN, USA), 0.1 per cent glucose and 1 per cent antibiotics (control serum-free media). Both cell lines were cultured in Primaria flasks which were coated with 0.44 μg/cm² fibronectin under SF conditions. In order to immunostain for TGF-α, JAr and BeWo were cultured to near confluence in serum-free media, trypsinized (2 × trypsin) and centrifuged at 2000 g. The pellets were fixed with 3 per cent p-formaldehyde (Sigma) for 2 h at room temperature (RT), embedded in paraffin blocks and 5 μm sections were cut and mounted on poly-L-lysine coated slides. Additionally, archived specimens of choriocarcinoma were obtained from the Department of Pathology at Northwestern Memorial Hospital in Chicago.

A431 epidermoid carcinoma cells were cultured in DMEM, 10 per cent FBS and 1 per cent antibiotics in 75-cm² Primaria flasks.

**3H-thymidine uptake**

The 3H-thymidine assay performed was similar to Shipley (1986) Benign CT at passage 4–7 or choriocarcinoma cells (BeWo or JAr) were plated into 24-well Primaria (Falcon) in standard media at 1.0–2.0 × 10⁴ cells/ml/well. The cells were weaned into SF media alone or SF media containing 0.5–50.0 ng/ml TGF-α (Intergen, Purchase, NY, USA). After 15–16 h the cells were labelled with 1 μCi/ml [methyl-3H]-thymidine (Dupont NEN, Wilmington, DE, USA; 20 mCi/mmol) for 2 h. The rest of the procedure was as described. Statistical analysis of the data was by a one-tailed Student’s t-test (Instat 2.01 statistical software, Graphpad Software).

**Cell proliferation assays**

Benign CT. All proliferation assays using benign and malignant cells were performed in 24-well plates. CT at passage 4–7 and originally isolated from placentae at 8 or at 10.5 weeks MA were plated at 1.0–1.5 × 10⁴ cells/well in F12/DME, 10 per cent FBS, 0.1 per cent glucose plus antibiotics (standard media). Cells were weaned into low serum media (F12/DME, 1 per cent FBS, 1.8 per cent TCH defined media supplement, 0.1 per cent glucose plus antibiotics) and after 24 h in low serum media day 0 counts were obtained. The remaining cells were refed with low serum media alone or with 0.5–50.0 ng/ml TGF-α. After 4 days cells were counted using a Coulter counter. Statistical analysis was by a one-tailed Student’s t-test.

JAr choriocarcinoma cells. JAr cells were plated at a density of 3.0 × 10⁴ cells/well in standard media and allowed to attach overnight. Day 0 counts were obtained the next day, and the remaining cells were refed with either standard media or standard media plus 0.1, 0.2 or 0.3 μg/ml neutralizing antibodies EGF Ab-2 or 1G1'α-α Ab-3 (clones 144-48 and 189-2130-I, respectively; Oncogene Science). Cells were counted each day for 4 days. In separate experiments JAr cells, adapted to continuous culture in SF media (JArSF), were plated into Primaria coated with 2 μg bovine fibronectin (Sigma). Cells were refed with either control SF media or media containing 0.2, 0.3 or 0.4 μg/ml EGF Ab-2 or TGF-α Ab-3 and counted on days 1–3, 5 and 7. Alternatively JArSF cells were plated at a density of 10⁴ cells/well and grown for 3 days in 0.7 or 1.6 μg/ml TGF-α Ab-3 and counted. JArSF cells were also
cultured for 3 or 5 days in control SF media or in control SF media plus 0.5, 0.25 or 1.0 μg/ml EGFR Ab-1 (clone 528, Oncogene Science) and counted. Finally, JArSF cells were cultured in control SF media for 3 days in the presence of 0, 10 or 20 ng/ml EGF or TGF-α respectively. Statistical analysis was by one-tailed Student’s t-test.

In order to calculate mean doubling times the following equation was used:

\[ T/3.33 \log_{10}(N/N_0) \]

where \( T \) equals the total time of the experiment in hours; \( N_0 \) equals the number of cells on day 0 of the experiment and \( N \) equals the number of cells at the end of the experiment.

Extraction of native membrane proteins

Native membrane proteins to be analysed for tyrosine kinase activity and EGFR quantitation were extracted using the method of Von Knebel Doeberitz et al. (1989) with modifications. Near confluent cultures of benign CT (passage 3) or choriocarcinoma cells were grown in control SF media for 48 h before membrane harvesting and then washed 2 × with ice cold phosphate buffered saline (PBS). Three millilitres of cold hypotonic lysis buffer containing protease inhibitors (10 mM Tris–HCl, pH 8.0, 10 mM KCl, 5 mM MgCl\(_2\), 1 mM phenylmethylsulphonylfluoride (PMSF) (Sigma), 2 μg/ml aprotinin (Intergen) and 2 mM [ethylenbis(oxycetylenitrilo)]tetraacetate acid [EGTA (Sigma)] were added and the cells were harvested by scraping. Lysates were transferred to 15-ml conical tubes and kept on ice for 30–45 min. Lysates were subjected to three cycles of snap freezing and thawing to disrupt cell membranes, and nuclei were pelleted at 4000 g for 10 min at 4°C. Supernatants were transferred to 2.0 ml ultracentrifuge tubes (Beckman) and centrifuged at 45 000 g for 40 min at 4°C; the membrane pellets were resuspended in either PBS [for enzyme-linked immunosorbent assay (ELISA) analysis] or 0.1 M HEPES (for tyrosine kinase activity assay) plus protease inhibitors. Protein concentrations were determined according to Bradford (1976), and membrane aliquots were stored at −80°C until analysed for EGFR quantitation or receptor tyrosine kinase activity.

Receptor binding assays

EGFR levels in A431 cells were determined by receptor binding assay. Membranes from this cell line were subsequently used to generate standard curves in an EGFR ELISA (see below). A431 cells were plated into 24-well Primaria at 3.0–6.0 × 10\(^4\) cells/well in DME, 10 per cent FBS, and antibiotics. After weaning the cells into SF F12/DME and cultured them for 48 h there were approximately 1.5–3.0 × 10\(^4\) cells/well. The rest of the assay was performed as described elsewhere (Frolik et al., 1984). Receptor binding data were analysed using Ligand 3.10 software developed by Munson and Robard (1980) to determine both receptor number and affinity. Analysis of the data indicated that the cells expressed approximately 8.5 × 10\(^6\) EGFR/cell (data not shown). As these cells had been cultured in SF media before receptor quantitation it is not unreasonable to find such high numbers of receptors. Additionally, Kawamoto et al. (1984) reported clonal A431 lines that expressed levels of EGFR within this range. Receptor affinities (1.99+0.20 × 10\(^{-9}\) M) were also within the range of those reported elsewhere (Kawamoto et al., 1983 and 1984).

ELISA analysis of native cell membranes

Test membrane (125 ng of protein/well) was added in 50 μl of carbonate buffer. Standard curves were generated from serial dilutions of A431 cell membranes. Membranes were incubated overnight at 4°C. EGFR ELISA was performed using the Immunoselect IEI and ELISA amplification systems using the protocol of the manufacturer. The primary antibody was EGFR Ab-1; τp e Ab-1 or β galactosidase Ab-1 were used as negative control antibodies. All antibodies were diluted 1 : 1000. Membranes isolated from the EGFR negative SU6 B-cell lymphoma line were used as a negative control.

EGFR tyrosine kinase activity assays (RTK)

RTK activity assays were performed according to Defize et al. (1986) with modifications. Twenty micrograms of A431 and 60 μg of BeWo or JAr membrane protein were diluted in 2 mM MnCl\(_2\), 10 mM p-nitrophenylphosphate, 80 μM sodium vanadate, 0.01 per cent bovine serum albumin (BSA) and 0.2 per cent Nonidet P-40 plus or minus 1 μM EGF. The final concentration of HEPES was 20 mM. Membranes with or without EGF were incubated at RT for 25 min. Fifteen micromolar ATP (Sigma) and 2 μCi γ\(^{32}\)P-ATP (3000 Ci/ mmol, Amersham) were then added to a final volume of 40 μl, and the samples were incubated on ice for 12 min. The reaction was stopped by boiling the samples in polyacrylamide gel electrophoresis (PAGE) sample buffer (1 mM Tris–HCl, pH 7.0, bromphenol blue, 20 per cent sodium dodecylsulphate (SDS), β-mercaptoethanol and 50 per cent sucrose: 1 : 1 : 2 : 1 : 2) for 5 min. Samples were electrophoresed on a 5.0 per cent acrylamide gel under denaturing conditions; gels were dried for 1 h at 80°C and exposed to Hyperfilm (Amersham) with intensifying screens for 20–22 h at −70°C.

Immunoperoxidase (IP)

IP was performed on formalin-fixed tissue and p-formaldehyde-fixed cells using a mouse Vectastain Elite ABC kit following the manufacturer’s procedure with modifications. Five per cent powdered milk in PBS was used as a blocking agent. TGF-α was localized using mouse monoclonal
antibody Ab-2 (clone 213-4.4, Oncogene Science), and mouse non-immune serum (Dako Industries, Carpenteria, CA, USA) served as a negative control. Both were diluted 1:33 before incubating overnight at 4°C with the tissue sections/cells. Following the diaminobenzidine (Dako) chromogenic reaction slides were counterstained with Harris haematoxylin (Sigma). Sections incubated with mouse non-immune serum demonstrated no appreciable staining (data not shown).

**Preparation of conditioned media (CM)**

CM was collected from cultures of BeWo and JAr, adapted to grow in SF media, at varying degrees of confluency after which cell numbers were determined. After centrifugation at 2000 g at 4°C for 10 min, PMSF was added to 1 mM and the CM was stored at −70°C. CM was concentrated 20 × using an Omegacell stirred cell with a 5-kDa molecular cut-off (MWCO) membrane (Filtron, Northborough, MA, USA) or 10 × using a Molecular/Por stirred cell with a 5-kDa MWCO type C membrane (Spectrum, Houston, TX, USA).

**Analysis of CM by ELISA**

ELISA analysis of CM from cultures of BeWo and JAr for TGF-α was performed with the Immuno Select and ELISA Amplification systems (Gibco BRL) as previously described (Filla, Zhang and Kaul, 1993).

**RESULTS**

**Effect of exogenous TGF-α on ³H-thymidine uptake of benign CT and BeWo and JAr cell lines**

³H-thymidine incorporation and stimulation of cell proliferation was assessed in cultures of benign CT isolated at 8 and 10.5 weeks MA treated with varying concentrations of TGF-α [Figure 1(a) and (b)]. Cells from both isolates demonstrated a mitogenic response to the growth factor although the 8 week isolate appeared to exhibit a stronger response compared with the 10.5 week isolate. It is reasonable to believe that the increase in thymidine uptake [Figure 1(a)] represents a true mitogenic response as opposed to an abortive attempt at proliferation as TGF-α also stimulated an increase in cell numbers in parallel experiments [Figure 1(b)]. The basal proliferation rate of the 8-week cells was higher than that of the 10.5-week cells [compare untreated cells, Figure 1(b)] possibly reflecting a greater proliferative capacity of the 8-week isolate, therefore these cells would exhibit a more robust response to growth factor. We thus confirmed our previous results (Filla, Zhang and Kaul, 1993) demonstrating that TGF-α is mitogenic for cultures of benign CT.

We extended our observations to malignant CT and examined the effects of TGF-α, and to a limited extent EGF, on ³H-thymidine uptake and/or proliferation in BeWo and JAr choriocarcinoma cells. ³H-thymidine uptake by BeWo was weakly stimulated by TGF-α (Figure 2(a)) but proliferation of this cell line was not significantly increased in the presence of 10 and 20 ng/ml EGF or TGF-α (Figure 2(b)). The results demonstrate that exogenous TGF-α is mitogenic in benign CT while weakly or not at all mitogenic in choriocarcinoma cells.
We did not compare the proliferation of benign CT with that of either choriocarcinoma cell line in a single experiment. However, by comparing the mean doubling times of both cell types during the course of each individual experiment [Figures 1(b) and 2(b), respectively] it can be seen that the proliferation rate of the JAr line is significantly greater than that of either benign CT isolate. The mean doubling time of the untreated 8-week benign CT during the experiment shown in Figure 1(b) was 44 h while that of the 10.5-week CT was 128 h. In comparison the mean doubling time of the untreated JAr cells during the experiment shown in Figure 2(b) was 36 h.

Quantitation of EGFR levels in benign and malignant CTs

Experiments were next undertaken to determine whether differences in the EGFR might account for the decreased mitogenic response to TGF-α exhibited by the choriocarcinoma cells. We developed an indirect ELISA to quantitate EGFR levels in membranes isolated from benign and malignant CT cultured in SF media (Table 1). Benign CT isolated from 8- and 10-week MA placenta expressed approximately 3.0–7.0 x 10^5 receptors/cell. BeWo and JAr cells expressed approximately 8.0 x 10^5 receptors/cell; these levels are over 10-fold greater than the levels exhibited by benign cells. Receptor affinities were not determined. The significant EGFR overexpression exhibited by BeWo and JAr would be expected to result in a strong mitogenic response to exogenous TGF-α or EGF. The observed weak response suggested that these receptors may not be functioning properly due to a mutation in the receptor itself or else due to the improper functioning of downstream effectors of the receptor.

EGFR RTK activity in BeWo and JAr

A functional RTK domain is essential for EGF and TGF-α mediated growth stimulation in responsive cells (Honegger et al., 1987). The functional state of the EGFR RTK in BeWo and JAr was assessed in isolated surface membranes from the two cell lines. Both BeWo and JAr membrane preparations possess a 170-kDa protein that demonstrates significantly increased incorporation of 32P in response to EGF (Figure 3). This band suggests a functional EGFR as it co-migrates with an intensely labelled protein of identical molecular weight previously identified as the EGFR in A431 cells (Buss, Chouvet and Gill, 1984). The results indicate that both cell lines possess a functional EGFR T capable of responding to exogenous EGF and TGF-α. Additionally, the results suggest that the overexpressed receptors are capable of properly binding ligand.

TGF-α immunostaining of BeWo and JAr and metastatic choriocarcinoma

Sections made from fixed pellets of BeWo or JAr cells, adapted to continuous culture in serum free media, were immunostained for TGF-α. Both cell lines demonstrated strong positive TGF-α immunostaining [Figure 4(a)] which demonstrates that choriocarcinoma cells are capable of making TGF-α in vitro.

Formalin-fixed sections of two specimens of metastatic choriocarcinoma in the lung were immunostained for TGF-α in order to determine if choriocarcinoma cells produced growth factor in vivo as well. As shown, the growth factor is localized to clumps of choriocarcinoma cells scattered throughout the lung tissue [Figure 4(b)]. These data are consistent
not correlate with the malignant behaviour of trophoblastic cells. Additionally, TGF-α levels in choriocarcinoma CM correlated inversely with the density of the cultures from which the CM was obtained; thus, a down-regulation of TGF-α production occurred as the cultures neared confluency. We reported similar findings in examining TGF-α production by cultures of benign CT (Filla, Zhang and Kaul, 1993), and others have reported this phenomenon in cultures of other benign and malignant cell types (Ferriola et al., 1992; Zorbas and Yeoman, 1993).

### Table 1. EGFR levels in benign and malignant trophoblasts

<table>
<thead>
<tr>
<th>Cell</th>
<th>EGFR (10⁵/cell)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign CT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 weeks</td>
<td>0.27 ± 0.10</td>
<td>6</td>
</tr>
<tr>
<td>8–10 weeks</td>
<td>0.71 ± 0.30</td>
<td>6</td>
</tr>
<tr>
<td>Malignant CT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BeWo</td>
<td>7.97 ± 1.73</td>
<td>9*</td>
</tr>
<tr>
<td>JAr</td>
<td>8.04 ± 1.67</td>
<td>9*</td>
</tr>
</tbody>
</table>

Samples were incubated at 50°C for 15 min before enzyme-linked immunosorbent assay (ELISA) analysis to reduce endogenous non-placental alkaline phosphatase (PAP) activity (Goldstein and Harris, 1979; Goldstein, Rogers and Harris, 1980). Standard curves were generated using serial dilutions of A431 membranes. Phenylglycine peptide (5 mM) was present in the ELISA-AMP adenosine monophosphate substrate solution in order to inhibit endogenous PAP (39,40). Statistical analysis was by a one-tailed t-test. * Significantly > first trimester cells, P=0.05.

### Effect of EGFR blocking antibody and EGF and TGF-α neutralizing antibodies on JAr choriocarcinoma proliferation

Further studies were performed to determine if choriocarcinoma cells were stimulating their own proliferation via a TGF-α-EGFR autocrine loop. Attempts to disrupt any interaction between EGFR and autologously produced ligand were made by culturing JAr cells in the presence of absence of an EGFR blocking antibody or neutralizing antibodies to TGF-α or EGF. Proliferation of JAr in SF media was significantly inhibited after a 3-day exposure to 0.25 and 1.0 μg/ml EGF Ab-1 [Figure 5(a)]. Only the highest EGFR Ab-1 concentration, 1.0 μg/ml, inhibited JAr proliferation after 5 days in culture; at lower antibody concentrations there was no effect [Figure 5(a)]. To confirm that the inhibition of proliferation was due to the disruption of TGF-α, rather than EGF, binding to the EGFR, JAr cells were cultured either in the presence or absence of EGF or TGF-α neutralizing antibodies. Increasing concentrations of EGF Ab-2 had no effect on JAr proliferation. In contrast, increasing concentrations of TGF-α Ab-3 significantly inhibited JAr proliferation [Figure 5(b) and (c)] in serum free media; this inhibition appeared to plateau at 20–30 per cent of control proliferation using antibody concentrations that ranged from 0.3–1.6 μg/ ml. Slightly lower TGF-α Ab-3 concentrations inhibited JAr proliferation when similar experiments were performed in the presence of 10 per cent FBS (data not shown). This degree of inhibition was also observed in the presence of EGFR blocking antibody concentrations that ranged from 0.25–1.0 μg/ml. The data confirm that JAr cells produce TGF-α and that an operational TGF-α–EGFR autocrine loop exists in these cells.

### DISCUSSION

We have previously shown that in vivo EGFR and TGF-α co-localized to benign CT throughout gestation while in vitro, cultured CT responded mitogenically to exogenous TGF-α and produced significant quantities of the growth factor (Filla, Zhang and Kaul, 1993). Thus we proposed that a TGF-α–EGFR autocrine loop stimulated the proliferation of these cells. Here we have confirmed our previous findings and extended our observations to choriocarcinoma cells in order to...
determine if alteration in the components of this regulatory loop might contribute to the malignant behaviour of trophoblastic cells. Two choriocarcinoma cell lines, BeWo and JAr (reviewed in Ringler and Strauss III, 1990) were used in our studies. In contrast to the proliferative response that we observed in benign CT, BeWo and JAr demonstrated a negligible to weak mitogenic response to exogenous TGF-α or EGF similar to previous reports (Huot et al., 1981; Ilekis and Benveniste, 1985; Filla et al., 1991; Lysiak, Graham and Lala, 1992). In vivo Miyachi et al. (1990) found low doses of EGF to stimulate growth of transplanted human choriocarcinoma tumours in nude mice while high doses of EGF inhibited tumour growth. In vitro EGF has been found, however, to induce a pseudodifferentiated phenotype in choriocarcinoma cell lines (Huot et al., 1981; Ilekis and Benveniste, 1985).

Several factors could account for the decreased mitogenic response to exogenous growth factor reported here, including alterations in the number and/or functional state of the EGFR, increases in the amount of an autologously produced EGFR ligand or acquisition of independence of the TGF-α–EGFR growth stimulatory pathway among others. Alternatively, it is possible that the lack of an observed mitogenic response in choriocarcinoma cells in the experiments described here reflects the induction of signal transduction pathways involved in differentiation rather than mitogenesis.

Using an EGFR ELISA we quantitated and compared receptor levels in benign and malignant CT and found a 10-fold overexpression of the receptor in the latter despite a decreased mitogenic response to TGF-α and EGF. Elevations of EGFR have been observed in malignant cells relative to their normal counterparts (Derynck et al., 1987; Di Marco et al., 1989). The significant EGFR overexpression exhibited

---

**Figure 4.** TGF-α immunostaining in: (a) BeWo choriocarcinoma cells adapted to continuous culture in serum free media; and (b) metastatic choriocarcinoma to the lung. Bar= 100 μm.
Table 2. TGF-α production in benign and malignant trophoblasts in serum-free media

<table>
<thead>
<tr>
<th>Density (10^5 cells/cm)</th>
<th>TGF-α (µg/ml/10^5 cells)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>BeWo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.16</td>
<td>3.41 ± 1.39</td>
<td>5*</td>
</tr>
<tr>
<td>0.76</td>
<td>1.68 ± 0.80</td>
<td>3</td>
</tr>
<tr>
<td>1.52</td>
<td>0.85 ± 0.40</td>
<td>11**</td>
</tr>
<tr>
<td>JAr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.23</td>
<td>2.52 ± 0.08</td>
<td>3*</td>
</tr>
<tr>
<td>1.30</td>
<td>1.64 ± 0.35</td>
<td>3</td>
</tr>
<tr>
<td>2.00</td>
<td>0.07 ± 0.29</td>
<td>17**</td>
</tr>
</tbody>
</table>

Conditioned media (CM) samples were incubated at 60°C for 15 min before enzyme-linked immunosorbent assay (ELISA) analysis to eliminate endogenous non-placental alkaline peroxidase (PAP) activity (Goldstein and Harris, 1979; Goldstein, Rogers and Harris, 1980). Negative controls consisted of incubating CM samples and serum free stock media with trp e Ab-1 or β-galactosidase Ab-1 or mouse non-immune serum or incubating stock serum free media with TGF-α Ab-1. PhGlyGly peptide (1111~) was present in the ELISA adenosine monophosphate (AMP) substrate solution in order to inhibit endogenous PAP (Goldstein and Harris, 1979; Goldstein, Rogers and Harris, 1980). Statistical analysis was by a one-tailed t-test. * Significantly > other cultures in group, P=0.05; ** significantly < other cultures in group, P=0.05.

by choriocarcinoma cells was unexpected given the weak mitogenic response to exogenous TGF-α and EGF observed in these cells. It is reasonable to believe that overexpression of the receptor by itself would result in a greater mitogenic response in BeWo and JAr relative to benign CT.

We next examined the functional state of the RTK of choriocarcinoma EGFR in order to determine whether or not the overexpressed receptors were able to transduce a ligand activated signal. EGFR RTK activity is essential for EGF and TGF-α mediated growth stimulation in cells responsive to these growth factors (Honegger et al., 1987), and a defective RTK domain in choriocarcinoma cell EGFR could account for the weak mitogenic activity of TGF-α in these cells. This possibility was eliminated after BeWo and JAr surface membrane preparations incubated with γ32P-ATP in the presence or absence of EGF demonstrated that the endogenous EGFR are capable of properly binding ligand and exhibit ligand dependent activation. This indicates that the overexpressed receptors are able to transduce TGF-α or EGF mediated signals. This does not, however, preclude the possibility that mediators of TGF-α–EGFR signal transduction pathways downstream of the receptor are functioning properly in choriocarcinoma cells.

We investigated the possibility that BeWo and JAr exhibited altered autologous production of TGF-α relative to benign CT. Both cell lines were adapted to continuous culture in SF media, and IP techniques demonstrated that they are able to produce TGF α. We also localized the growth factor in vivo in CT elements of specimens of metastatic choriocarcinoma. This indicates that BeWo and JAr did not newly acquire the ability to produce TGF-α as the two cell lines adapted to growth in culture because we have previously demonstrated TGF-α production by benign CT (Filla, Zhang and Kaul, 1993). We did not localize EGFR in these specimens as it has been well documented that choriocarcinoma cells express EGFR (Ilekis and Benveniste, 1985; Miyachi et al, 1990; Ladines-Llave et al., 1993).
We quantitated levels of TGF-α in BeWo and JAr SF CM in order to determine whether overproduction of the growth factor correlated with choriocarcinoma EGFR overexpression relative to benign CT. Others have reported a correlation between the absence of a mitogenic response to EGF or TGF-α in the transformed counterparts of cells that are normally responsive to these growth factors and increased autologous TGF-α production (Salomon et al., 1987; Ciardiello et al., 1988). Although TGF-α levels in choriocarcinoma CM were found to be higher than those reported elsewhere for other benign and malignant cell types (Coffey et al., 1987; Skinner et al., 1988; Imanishi et al., 1989; Ferriola et al., 1992; Zorbas and Yeoman, 1993), they were similar to levels in CM from benign CT reported in our previous study (Filla, Zhang and Kaul, 1993). Our results are in agreement with those of Bissonnette et al. (1992) who found that levels of TGF-α extracted from whole placentae were five- to 30-fold higher than those reported for benign and malignant tissues elsewhere.

In order to demonstrate autocrine stimulation of choriocarcinoma proliferation via a TGF-α-EGFR regulatory loop, we attempted to disrupt EGF ligand-receptor interactions in JAr cells by culturing them in the presence of TGF-α or EGF neutralizing antibodies (TGF-α Ab-3 and EGF Ab-2, respectively) or an EGF blocking antibody (EGFR Ab-1). Proliferation of JAr in SF media was significantly inhibited in the presence of EGFR Ab-1 which blocks the binding of both TGF-α and EGF to the receptor (Kawamoto et al., 1983, 1984). TGF-α Ab-3 inhibited JAr proliferation while EGF Ab-2 had no effect suggesting that EGFR Ab-1 was inhibiting the binding of autologously produced TGF-α rather than EGF. It is possible that higher concentrations of EGF Ab-2 would have resulted in the inhibition of JAr proliferation as this cell line has been shown to express low levels of EGF mRNA (Steller et al., 1994). Given that EGF Ab-1 and TGF-α Ab-3 inhibited JAr proliferation we propose that a TGF-α-EGFR autocrine loop contributes to stimulating the proliferation of these cells. Further experiments are required to determine whether or not such a loop regulates choriocarcinoma cell proliferation in general.

Although JAr proliferation was inhibited by 20–30 per cent in the experiments described above, a greater degree of growth inhibition had been expected. It is possible that TGF-α—EGFR juxtacrine (Anklesaria et al., 1990; Zorbas and Yeoman, 1993) and/or intracrine (Kennedy, Brown and Vaughan, 1993; Sherman et al., 1993) stimulatory loops may also be operating in JAr cells. A significant fraction of the TGF-α produced by the cells or the EGF expressed at the cell surface might, therefore, have been inaccessible to either TGF-α Ab-3 or EGF Ab-1, respectively. Further studies would be required to explore these possibilities in greater detail.

The data presented here and in our previous report (Filla, Zhang and Kaul, 1993) indicate that a TGF-α—EGFR autocrine loop may regulate the proliferation of both normal and malignant CT. In malignant CT, however, this regulatory loop appears to be deranged as evidenced by the failure of exogenous TGF-α to stimulate choriocarcinoma proliferation despite the presence of functional receptors. Choriocarcinoma cell lines, relative to benign CT, appear to exhibit dysregulated responses to TGF-β (Graham et al., 1994) and tumour necrosis factor-α (TNF-α) (Yang et al., 1993; Yui et al., 1994) as well. JAr and JEG-3 have both become refractive to both the anti-proliferative and anti-invasive activity of TGF-β while exhibiting proliferative rather than apoptotic responses to TNF-α. These dysregulated responses would offer clear growth advantages to transformed cells relative to their benign counterparts while the dysregulated response of BeWo and JAr to TGF-α described here does not. In contrast, their significant EGFR overexpression together with the significant quantities of TGF-α autologously produced by either cell line may play a role in their rapid and uncontrolled proliferation and thus make them unresponsive to further stimulation by exogenous TGF-α or EGF. Further studies are required to clarify the role(s) of TGF-α—EGFR interactions in regulating the behaviour and function of the normal placenta and the development of trophoblastic neoplasms.

ACKNOWLEDGEMENTS

This work was supported in part by the Carcinogenesis Training Program, National Cancer Institute Grant T32CA09560 (M.S.F.), and by grants from the Schweppe Foundation and the Cancer Research Foundation (K.L.K.). Portions of this work have appeared previously as part of a doctoral dissertation (M.S.F.).

The authors would like to acknowledge the help of Dr Diane Boucher (Northwestern University Medical School) in the preparation of the figures, Dr Phil Iannaccone (Northwestern University Medical School) who shared laboratory space and equipment, and Gloria Clay (Evanston Hospital) for expert secretarial assistance.

REFERENCES


Steller, M. A., Mok, S. C-H., Yeh, J., Fulop, V., Anderson, D. J. & Berkowitz, R. S. (1994) Effects of cytokines on epidermal growth factor...


