Immunohistochemical localization of insulin-like growth factors I and II at the primary implantation site in the Rhesus monkey

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There are various cellular mediators which can affect the process of blastocyst implantation by regulating the proliferation and differentiation of conceptus and maternal endometrial cells. Insulin-like growth factors I (IGF-I) and II (IGF-II) are potent mitogenic and differentiation-promoting growth factors. However, the role of IGF peptides at implantation in primate species is not well understood. The objective of the present study was to immunohistochemically localize IGF-I and IGF-II peptides in trophoblast cells and maternal endometrial cells during lacunar and villous stages of placentation in the Rhesus monkey. Female animals (n=10) were laparotomized on estimated days 13-16 after fertilization to collect primary implantation sites which were subjected to immunohistochemical staining for IGF-I and IGF-II peptides. Cell-type specificity for IGF-I and IGF-II was evident with a very low level of IGF-I peptide immunolocalized in trophoblast cells lining lacunae, and primary and secondary villi, while moderate to high amounts of IGF-II peptide were detected in lamellar syncytiotrophoblast cells lining lacunae, early villi and cell columns, as well as in migrating trophoblast cells in the extravillous compartment and in endovascular trophoblast cells. The observed presence of IGF-II peptide in differentiated lamellar syncytiotrophoblast cells during the very early stages of implantation and placentation in the Rhesus monkey may be important in their transition to this differentiated cell population. Maternal endometrial cells showed similar distribution profiles for IGF-I and IGF-II. In conclusion, we report differential distribution of IGF-I and IGF-II peptides in trophoblast cell populations at the feto-maternal interface during lacunar and villous stages of gestation in the Rhesus monkey.

Key words: cytotrophoblast/endometrium/implantation/insulin-like growth factors/syncytiotrophoblast

Introduction

The cellular events at implantation and placentation in primate species have been studied using the macaque and the baboon (Hendrickx, 1971; Enders and Schlafke, 1986). A study of the early stages of implantation revealed that penetration of uterine epithelium by syncytial trophoblast cells occurs on day 9.5 of gestation followed by marked proliferation of cytotrophoblast cells which form the trophoblast plate (Enders et al., 1985). Endothelial basal lamina are penetrated by cytoplasmic processes arising from syncytial trophoblast cells, and at this time syncytial clefts and lacunae lined by polarized lamellar syncytiotrophoblast cells also begin to develop (Enders and King, 1991). By day 12 of gestation, lacunae undergo further development and become filled with maternal blood as syncytiotrophoblast cells penetrate maternal blood vessels. The responses of maternal endometrium during trophoblast penetration is characterized by marked oedema in the upper functionalis and transformation of epithelial cells at neck of glands to form nests of plaque acini. Stromal cell decidualization along with an influx of endometrial granulated lymphocytes then begins by day 14 of gestation as plaque acini begin to degenerate (Enders et al., 1985).

The dynamic process of trophoblast invasion into maternal endometrium and vasculature is associated with extensive cell–cell and cell-matrix interactions involving various adhesion molecules and matrix metalloproteinases (Librach et al., 1991; Blankenship et al., 1993a,b; Enders and Blankenship, 1997). However, the cellular mediators which can affect the process of blastocyst implantation during the early stages of gestation remain largely unknown. Immunohistochemical analyses of receptors for oestradiol-17β (ER) and progesterone (PR) at fetal-maternal interface in timed pre-villous and villous stages of implantation of Rhesus monkey revealed the presence of PR in syncytiotrophic parts of the cells, while these cells were generally ER negative. These analyses also showed that maternal endometrial cells exhibited heterogeneous staining...
patterns for ER and PR, and this correlated well with endometrial hyperplasia, differentiation and stromal-decidual transformation (Ghosh et al., 1999).

Insulin-like growth factor I (IGF-I) and IGF-II are potent mitogenic and differentiation-promoting growth factors (Clemens, 1991). There is robust evidence to support the importance of IGF in the regulation of fetal growth and development in mice (Baker et al., 1993; Liu et al., 1993). IGF are involved in the growth and differentiation of trophoblast cells both in vitro (Fant et al., 1986; Ritvos et al., 1988) and in vivo (Guidice et al., 1995; Coulter and Han, 1996). In a study of human placental tissue recovered between 6 and 8 weeks of gestation, IGF-II was shown to be abundantly expressed by invading cytotrophoblast cells (Han et al., 1996). IGF-II has also been shown to stimulate human trophoblast in vivo (Irving and Lala, 1995). However, none of these studies investigated the involvement of IGF peptides at implantation sites during early stages of implantation and placentation in a primate species. The objective of the present study was to immunohistochemically localize IGF-I and IGF-II peptides in trophoblast cells and in maternal endometrial cells during lacunar and early villous stages of placentation in the Rhesus monkey.

Materials and methods

Animals

Proven fertile male and female Rhesus monkeys were housed singly under semi-natural conditions in the Primate Research Facility of the All India Institute of Medical Sciences, and were fed with a regular monkey pellet diet, semi-formulated Indian bread, fresh seasonal fruits and water ad libitum. Females were allowed to cohabit with their male partners during days 8-16 of their menstrual cycles and during this time peripheral blood samples were collected once daily from female monkeys for the determination of oestradiol-17β and progesterone in peripheral circulation by radioimmunoassays in order to detect the day of ovulation (day 0) and for the determination of chorionic gonadotrophin (mCG) levels in peripheral circulation by radioimmunoassays in order to detect the day of ovulation (day 0) and for the determination of chorionic gonadotrophin (mCG) levels in peripheral circulation by radioimmunoassays in order to detect the day of ovulation (day 0) and for the determination of chorionic gonadotrophin (mCG) levels in peripheral circulation. Cytokines and vimentin using specific monoclonal antibodies (Dako-CK MNF116 and Dako-vimentin V9, respectively) from Dako A/S (Glostrup, Denmark). All other chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA). Sections were incubated with primary antibody overnight at 4°C, followed by incubation with biotinylated secondary antibody. Final visualization was achieved using the ABC peroxidase kit (Vector Laboratories, Burlingame, CA, USA) and freshly prepared 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide according to the protocol provided by the manufacturer. Sections were then counterstained lightly with haematoxylin.

Dilutions of stock primary antibodies for incubation were pre-calibrated based on 3–5 point titration and the information provided by the manufacturer. Specificity of antibody ligand binding and visualization were assessed by: (i) omitting primary antibodies, (ii) replacing primary antibodies with unrelated immunoglobulin from the same species and other species, and (iii) omitting secondary antibodies and replacing labelled secondary antibody with unrelated labelled immunoglobulin from the same and other species. Specificities of the antibodies against IGF-I and IGF-II were further examined using immunoblotting and immunoneutralization on adjacent tissue sections. For a given antibody and dilution, all sections were subjected to immunohistochemistry simultaneously. Late proliferative and mid luteal phase human endometrial tissue sections were used as positive controls.

For the assessment of immunostaining in cells of fetal and endometrial compartments, semiquantitative subjective scoring was done by all three investigators separately using a standardized 5-scale system: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), 4 (76% and more), as described previously (Press et al., 1988; Ghosh et al., 1999). The immunostained sections which yielded coefficient of variance more than 10% in the pooled data analysis were not included. It was assumed that these measurements reflected the concentrations of the investigated proteins in fetal and maternal cells at blastocyst implantation sites.

Results

Figure 1A-F shows representative lacunar and early villous stages of implantation and placentation of the Rhesus monkey. Light microscopic examination of primary implantation sites revealed the presence of lacunae lined with syncytiotrophoblast cells and overlying the chorionic plate on day 13 of gestation. Between days 14 and 16 of gestation, expansion of
Figure 1. Micrographs of immunohistochemically stained sections for cytokeratin (A-C) and vimentin (D-F) in lacunar (A, D) and early villous (B, C, E, F) stages of implantation. Immunopositive cytokeratin staining is detected in trophoblast cells lining lacunae (L), primary villi (PV), secondary villi (SV) and trophoblast cells within the chorionic plate, as well as in interstitial trophoblast cells and endovascular trophoblast cells (arrow head). Immunopositive staining for vimentin (D-F) shows that trophoblast cell populations are negative, while extraembryonic mesodermal cells and maternal stromal cells beneath chorionic plate are positive. Bars = 100 µm.

Figure 2. Immunohistochemical staining for insulin-like growth factor (IGF)-I (A) and IGF-II (B) at the lacunar stage of implantation. Immunostaining for IGF-I was not detected in cytotrophoblast cells (ct) and syncytiotrophoblast cells (st) lining lacunae (L); however, occasionally large trophoblast cells (asterisk) adjoining the implantation site were distinctively positive. Distinctive immunostaining for IGF-II was found in syncytiotrophoblast cells (st) lining lacunae (L), while a lower degree of immunostaining was found in trophoblast cells of the plate region. Bars = 20 µm.

Figure 3. Immunohistochemical staining for insulin-like growth factor (IGF)-II in early villous stage of implantation. Syncytiotrophoblast cells (arrow heads) lining the cell column are positive, while non-polarized cells within the column (CC) are negative for IGF-II. A migratory extravillous trophoblast cell (arrows) shows strong immunopositive staining for IGF-II. Bar = 10 µm.
lacunae was observed followed by the formation of primary and secondary villi along with marked expansion of the implantation area. Endometrial responses to invading trophoblast included development of subepithelial oedema and formation of epithelial plaque acini adjacent to the implanting nidus. Throughout this period, venular dilatation and penetration of the vascular compartment by cytotrophoblast cells were remarkable features at the site of implantation.

As shown in Figures 1A-C, all populations of trophoblast cells including migrating trophoblast cells within endometrial stroma and glands and endovascular cytotrophoblast cells within dilated blood vessels were immunopositive for cytokeratin. Endometrial glandular epithelium and plaque acinar epithelium were also cytokeratin-positive. Vimentin-positive maternal endometrial stromal cells were observed surrounding dilated blood vessels and glands at implantation sites. Extraembryonic mesenchymal cells were also vimentin positive (Figures 1D-F), and they were also occasionally stained positive for cytokeratin.

Figures 2–5 show immunolocalization of IGF-I and IGF-II in different cell types at the fetal-maternal interface and in cells of the endometrial-myometrial compartment at lacunar and early villous stages of implantation collected on days 13–16 of gestation. Table I provides the corresponding scores for immunopositive IGF-I and IGF-II peptides in cells of fetal and endometrial compartments. At the lacunar stage, immunopositive IGF-I peptide was only occasionally detected in

**Table 1.** Immunohistochemical localization of insulin-like growth factor I (IGF-I) and IGF-II in the primary implantation sites of the Rhesus monkey

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Median score (ranges)(^a)</th>
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<tbody>
<tr>
<td></td>
<td>IGF-I</td>
</tr>
<tr>
<td>Lacunar CTB cells</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Lacunar STB cells</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Villous CTB cells</td>
<td>1 (0–2)</td>
</tr>
<tr>
<td>Villous STB cells</td>
<td>1 (0–2)</td>
</tr>
<tr>
<td>Non-polarized CTB in cell columns</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>STB lining of cell columns</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Extravillous CTB</td>
<td>1 (0–2)</td>
</tr>
<tr>
<td>Endovascular CTB</td>
<td>1 (0–1)</td>
</tr>
<tr>
<td>Luminal epithelium</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Glandular epithelium</td>
<td>1 (1–3)</td>
</tr>
<tr>
<td>Plaque epithelium</td>
<td>3 (2–4)</td>
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<tr>
<td>Fibroblast cells</td>
<td>1 (0–2)</td>
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<tr>
<td>Decidual cells</td>
<td>1 (1–3)</td>
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<tr>
<td>Endothelial cells</td>
<td>0</td>
</tr>
<tr>
<td>Vascular smooth muscle cells</td>
<td>3 (2–4)</td>
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<tr>
<td>Myometrium</td>
<td>3 (2–4)</td>
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\(^a\)Based on semiquantitative subjective scoring using a standardized 5-scale system: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), 4 (>76%).

CTB = cytotrophoblast; STB = syncytiotrophoblast.
imunohistochemical staining for insulin-like growth factor (IGF)-I (A, C) and IGF-II (B, D) in cells of superficial (A, B) and basal (C, D) endometrium of villous stage of implantation. Positive immunostains are seen in epithelial plaque acini (P) (A, B), vascular smooth muscle cells and myometrial cells (M) (C, D). Bars = 10 µm (A, B), 20 µm (C, D).

![Image](image-url)

**Figure 7.** Immunohistochemical staining for insulin-like growth factor (IGF)-I (A, C) and IGF-II (B, D) in cells of superficial (A, B) and basal (C, D) endometrium of villous stage of implantation. Positive immunostains are seen in epithelial plaque acini (P) (A, B), vascular smooth muscle cells and myometrial cells (M) (C, D). Bars = 10 µm (A, B), 20 µm (C, D).

Trophoblast cells associated with lacunae and in adjoining maternal interstitium (Figure 2A; Table I), while IGF-II immunostaining was predominant in lamellar syncytiotrophoblast cells lining the syncytial cleft and lacunae (Figure 2B; Table I). Trophoblast cells within the chorionic plate exhibited a lower level of immunostaining, while cells beneath and adjacent to lacunae remained largely negative for IGF-II (Figure 2B). The villous trophoblast cells showed very low to no immunostaining for IGF-I (Table I), while discrete IGF-II peptide was seen in villous syncytiotrophoblast cells (Figure 3). Although there was only occasional staining for IGF-I and IGF-II in non-polarized trophoblast cells of the cell columns, marked immunostaining for IGF-II was observed in syncytiotrophoblasts lining the cell column, in migrating extravillous trophoblast cells (Figure 4) and in endovascular trophoblast cells (Figure 5). Figure 6 shows the absence of immunopositive staining in fetal and maternal endometrial cells in the absence of primary antibodies. In the maternal compartment, a low to moderate level of immunostaining for both IGF-I and IGF-II was seen in plaque epithelial cells, glandular epithelial cells, vascular smooth muscle cells and myometrium in lacunar and villous stages of implantation (Table I; Figure 7A-D).

**Figure 8.** Immunobloting (A, B) and immunohistochemical (C-F) examination of specificity of antibodies against insulin-like growth factor (IGF)-I (A, C, D) and IGF-II (B, E, F) used. For immunoblot analysis, with pre-stained molecular markers (Amersham Life Sciences) (lanes 1 and 4), recombinant human (rh)IGF-I (100 ng/lane; lanes 2 and 5), and rhIGF-II (100 ng/lane; lanes 3 and 6) were subjected to 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes and immunodefected using antibodies (1.0 µg/ml) against IGF-I (A) and IGF-II (B). Arrows show immunopositive bands at mol. wt 7.5 kDa. Immunohistochemical neutralization (C-F) was performed with adjacent sections incubated with primary antibodies (5 µg/ml) against IGF-I (C, D) and IGF-II (E, F) in presence of rhIGF-II (C, F) and rhIGF-I (D, E). Bars = 40 µm.
Discussion

The observed light microscopic changes during lacuna and early villous stages of implantation and placentation at primary implantation stages in the Rhesus monkey were very similar to those reported earlier in the same species (Wislocki and Streeter, 1938; Heusser and Streeter, 1941; Enders et al., 1985; Enders and Schlafke, 1986; Enders, 1993). All cytotrophoblast and syncytiotrophoblast cell populations were cytokeratin-positive, though no effort was made to differentiate different subsets of trophoblast cells based on their patterns of cytokeratin expression as has been reported earlier for human trophoblast cell populations (Muhlhauser et al., 1995; Vicovac and Aplin, 1996).

It is generally known that IGF-I and IGF-II are potent mitogenic and differentiation-promoting growth factors and appear to act via paracrine and autocrine as well as classical endocrine mechanisms (Rotwein, 1991). We now report for the first time that IGF-I and IGF-II peptides are localized, in a cell-type-specific manner, during the very early stages of implantation and placentation in the Rhesus monkey. A low level of IGF-I was immunolocalized in trophoblast cells lining lacunae and primary and secondary villi, while moderate to high amounts of IGF-II were detected in lamellar syncytiotrophoblast cells lining lacunae, villi and cell columns, as well as in migrating trophoblast cells in the extravillous compartment, and in endovascular trophoblast cells. Similar observations have been made in monkey placenta collected during day 65 to term (Coulter and Han, 1996) and in human placental tissue (Han et al., 1996). Maternal endometrial cells, however, showed highly comparable distribution profiles for both IGF-I and IGF-II.

The observed presence of IGF-II peptide in differentiated lamellar syncytiotrophoblast cells during the very early stages of implantation and placentation in the Rhesus monkey may be important in their transition to this differentiated cell population, since it is known that IGF-II functions as an essential survival factor during transition from the proliferating state to the differentiating state (Stewart and Rotwein, 1996). It is possible that IGF-II regulates the role of syncytiotrophoblast cells in the placental functions of nutrient and oxygen transport as well as placental hormone synthesis and secretion (Roberts and Anthony, 1994; Coulter and Han, 1996). It has also been suggested that IGF-II produced by trophoblast cells within the choriionic plate, as has been observed in the present study, may potentiate their migratory invasion (Irving and Lala, 1995) and stimulate insulin-like growth factor 1 (IGFBP-1) production by decidual cells in a paracrine manner (Irwin et al., 1993). IGFBP-1 can in turn in

In conclusion, it appears that there is differential distribution of IGF peptides in syncytiotrophoblast, cytotrophoblast and migrating trophoblast cell populations in lacunar and villous stages of gestation in the Rhesus monkey. In order to appreciate the involvement of IGF in the complex process of trophoblast invasion and decidualization in the primate uterus it appears important to examine the IGF-IGFBP-1 involvement at fetomaternal interface in the primate.

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