Developmental regulation of integrin expression at the time of implantation in the mouse embryo

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SUMMARY

The trophectoderm layer of the mouse blastocyst differentiates at the late blastocyst stage to form the invasive trophoblast that mediates implantation of the embryo into the uterine wall. The first sign that trophoblast cells have developed an invasion-specific cell behavior appears about 10-15 hours after the embryo hatches from the zona pellucida, when the quiescent, non-adherent trophectoderm cells initiate protrusive activity and become adhesive to extracellular matrix. Our previous findings that trophoblast outgrowth on extracellular-matrix-coated substrata involves the integrin family of adhesion receptors (Sutherland, A. E., Calarco, P. G. and Damsky, C. H., 1988, J. Cell Biol. 106, 1331-1348), suggested that the onset of trophoblast adhesive and migratory behavior at the time of implantation may be due to changes in expression or distribution of integrin receptors. We have thus examined the mRNA and protein expression of individual integrin subunits during pre- and perimplantation development (E0-E7.5). A basic repertoire of integrins, including receptors for fibronectin (α5β1), laminin (α6β1) and vitronectin (αVβ3), was expressed continuously throughout this period, whereas the expression of five other integrin subunits was developmentally regulated. The mRNA for three of these (α2, α6β1 and α7) was first detected in the late blastocyst, coincident with endoderm differentiation and development of attachment competence. The mRNA for another (α11) was not detected until after trophoblast outgrowth had begun, suggesting that its expression may be induced by contact with matrix. At E7.5, three of the temporally regulated integrins (α11, α6β1, α7), all of which can form receptors for laminin, were detected only in the ectoplacental cone (differentiating trophoblast), and may thus play specific roles in trophoblast adhesion and/or differentiation.

Because laminin expression is upregulated in decidualized uterine stroma in response to the implanting embryo, we examined trophoblast-laminin interactions, using laminin fragments and integrin antibodies to determine which integrin receptors were involved. Trophoblast cells attached and spread on both the E8 and P1′ fragments of laminin; however, the P1′ binding site was cryptic in intact laminin. Interaction with P1′ was RGD- and α6β1-dependent, whereas outgrowth on E8 was RGD-independent and not inhibited by antibodies to the laminin receptor α6β1, suggesting that α6β1 is the major trophoblast integrin E8 receptor. In contrast, migration of parietal endoderm cells on laminin was blocked by antibodies to α6, demonstrating that these two contemporaneous migratory cell populations have different modes of interaction with laminin. We conclude that developmental regulation of integrin expression appears to have functional significance for trophoblast invasion of the laminin-rich uterine stroma.

Key words: cell-matrix interactions, implantation, integrins, laminin, mouse embryo, trophoblast

INTRODUCTION

After an initial period of cleavage and blastocyst formation, mammalian embryos must make contact with an external source of nutrients in order to grow and continue their development. The mouse embryo accomplishes this by implanting itself into the stroma of the uterus and forming contacts with the maternal blood supply that lead to establishment of a hemochorial placenta (Schlafke and Enders, 1975; Enders, 1976). Implantation of the embryo is mediated by a specialized population of cells, the trophoblast cells, which arise from the trophectoderm, the outer layer of epithelial cells that encloses the blastocoel cavity and the inner cell mass of the preimplantation blastocyst.

Conversion of the epithelial trophectoderm to invasive trophoblast begins at the late blastocyst stage, and comprises both a change in adhesive behavior and the onset of motility (Chavez, 1984; Bevilacqua and Abrahamsohn, 1988). Trophoblast differentiation and invasion can be modelled in vitro, using embryos cultured on substrata of extracellular matrix molecules (Jenkinson and Wilson, 1973; Arman et al., 1986; Sutherland et al., 1988). The ability to attach to such substrata is acquired during trophoblast differentiation; early blastocysts cultured under these conditions do not
adhere and spread (Chavez, 1984), whereas late blastocysts will form an embryo outgrowth, consisting of a monolayer of spreading trophoblast cells with the inner cell mass remaining on top. Time-lapse observations of embryo outgrowth cultures show that the first signs of outgrowth are detected about 10-15 hours after the embryo hatches from the zona pellucida, when the previously quiescent trophectoderm cells exhibit an abrupt onset of protrusive activity followed by attachment and spreading on the substratum (Sutherland et al., 1988).

Trophoblast interactions with extracellular matrix molecules depend largely on the integrin family of adhesion receptors, as broad spectrum anti-integrin antibodies will block trophoblast attachment and outgrowth on fibronectin, laminin or collagen IV-coated substrata (Sutherland et al., 1988). The changes in trophoblast adhesive and migratory behavior that occur at the time of implantation may therefore stem from changes in the expression or distribution of integrin receptors. To address this possibility, we examined the temporal and spatial expression patterns of integrin subunits, and tested the function of several integrins in attaching to laminin, an extracellular matrix molecule known to be a major component of the uterine environment. We found that, whereas five integrin subunits were expressed throughout preimplantation and early postimplantation stages, four other subunits were first expressed at the time of implantation, three of which appeared to be trophoblast-specific. Expression of the regulated subunits appears to have functional significance for implantation, as trophoblast interactions with laminin, whose expression in the uterine stroma is upregulated in response to the implanting blastocyst (Wewer et al., 1986; Glasser et al., 1987; Senior et al., 1988), are not mediated by integrin receptors continuously expressed prior to implantation.

MATERIALS AND METHODS

Antibodies

Polyclonal rabbit antisera to cytoplasmic domain peptides of α1, α2, and α5 subunits were the generous gift of Dr Louis Reichardt (UCSF). Polyclonal rabbit antiserum to a cytoplasmic domain peptide of α3 was the gift of Dr Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA). Polyclonal rabbit antiserum against αβ3, was the gift of Dr James Gailit and Dr Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). Polyclonal antiserum to α6 and the rat monoclonal antibody GoH3 against mouse α6 were gifts of Dr Arndt Sonnenberg (The Netherlands Cancer Institute, Amsterdam). The rat monoclonal antibody 346-11A against mouse α1 was the gift of Dr Steve Kennel (Oak Ridge National Laboratory, Oak Ridge, TN). The rat monoclonal antibody 5H10 against mouse α5 was purchased from Pharmingen Inc. (San Diego, CA). Laminin fragments E1′, E3, E4, E8 and P′1, and a recombinant form of the laminin A chain COOH-terminal globular domain (rG), as well as antibodies against these fragments were the generous gift of Dr Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ). Each anti-fragment antiserum had been affinity-purified over a column of the immunizing fragment, then cross-absorbed over columns of each of the other fragments. The specificity of each anti-fragment antibody had then been confirmed by Western blotting and competition ELISA experiments. Controls included nonimmune rabbit sera and the rat monoclonal antibody AIIIB2 against human β1, which does not recognize the mouse protein (Hall et al., 1990).

Materials

Mouse laminin and Mito+ were purchased from Collaborative Research (Bedford, MA). Mouse fibronectin and the hexapeptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Glu-Ser-Pro (GRGESP) were purchased from Telios (La Jolla, CA). Sepharose CL-4B and protein A-Sepharose were from Pharmacia (Piscataway, NJ); Expre35S35S protein labelling mixture and En3Hance were purchased from New England Nuclear-Dupont (Boston, MA). Bovine serum albumin (BSA), insulin, N-octyl-β-D-glucopyranoside, pancreatic, Tween 20 and the protease inhibitors phenylmethylsulfonylfluoride (PMSF), leupeptin and pepstatin were from SigmaChemicals (St Louis, MO). Acrylamide gel electrophoresis reagents were purchased from BioRad (Richmond, CA). Fetal bovine serum (FBS) was from the UCSF Cell Culture Facility. Prestained protein molecular weight standards were obtained from Bethesda Research Laboratories (Gaithersburg, MD), and included myosin (Mr 205×10^3), phosphorylase b (Mr 94×10^3), bovine serum albumin (Mr 68×10^3), ovalbumin (Mr 43×10^3) and carbonic anhydrase (Mr 29×10^3).

Embryo culture

Preimplantation embryos were flushed at the 2-cell stage from the oviducts of superovulated female ICR mice (12 weeks old; Harlan Sprague-Dawley Inc.) and cultured through the preimplantation stages of development as described previously (Sutherland et al., 1988). Late blastocysts were obtained by culturing embryos at the early blastocyst stage (3 days in culture from the 2-cell stage) for an additional 48 hours in serum-free Eagle’s medium (Spindle and Pedersen, 1973; Spindle, 1980) supplemented with insulin (10 µg/ml), BSA (4 mg/ml), and Mito+ (0.1%). Embryo outgrowths for use in polymerase chain reaction, immunoprecipitation and immunofluorescence experiments were obtained by culturing late blastocysts for an additional 24 hours in Eagle’s medium supplemented with 10% FBS. Ectoplacental cone (EPC) explants were obtained and cultured as described previously (Sutherland et al., 1991).

Embryo outgrowth on defined substrata

Antibody and peptide dilutions were prepared in CMRL medium 1066 (Gibco, Grand Island, NY) supplemented with 0.05 mg/ml sodium pyruvate, 4 mg/ml BSA, and 1% Nutridoma-HU. All media were filter-sterilized, using syringe-tip filters (Millipore, HV) of 0.45 µm pore size, before addition to the substrata. Endoderm outgrowth assays were done using Eagle’s medium supplemented with Mito+ and 2% FBS, as these were the only conditions under which parietal endoderm cells were produced reliably in vitro.

Substrata [laminin (25 µg/ml), fibronectin (20 µg/ml), laminin fragments E1′, E3, E4, E8, P′1 and rG (each at 25 µg/ml)] were prepared as described previously (Sutherland et al., 1988), and experimental media were added and equilibrated for 30 minutes at 37°C. Late blastocysts were then added (5-15 embryos per drop) and cultured at 37°C for 72-96 hours. Trophoblast outgrowth was assayed at 72 hours as described previously (Sutherland et al., 1988). Endoderm outgrowth was assayed visually at 96 hours by examining the embryos for the presence and distribution of small parietal endoderm cells. Each treatment was used in duplicate in at least three independent experiments.

Radiolabelling of embryos

Preimplantation embryos (4-cell to blastocyst stages) were incubated for 6-8 hours in medium containing 75 µCi/ml of Expre35S35S protein labelling mixture, lysed in 100 µl OGB [0.2 M N-octyl-β-D-glucopyranoside in PBS, containing the protease inhibitors PMSF (2 mM), pepstatin (1 µg/ml) and leupeptin (1
Immunoprecipitation
Preimplantation embryo lysates were pooled to obtain samples containing the equivalent of 1200-1500 embryos and lysates from 5-8 EPC explants were pooled. These pooled samples were then subjected to sequential immunoprecipitation. The lysates were spun in a refrigerated microfuge for 15 minutes, preabsorbed twice on pellets of Sepharose CL-4B and incubated with primary antibody overnight at 4°C. The samples were then added to 50 µl pellets of protein A-Sepharose beads for one hour, absorbed twice more on 50 µl pellets of protein A-Sepharose (30 minutes each), after which the next primary antibody was added to the lysates and incubated overnight. The protein A-Sepharose absorption protocol was repeated for each antibody used in the experiment, and each lysate was treated with 3-5 different primary antibodies. The first and third sets of protein A-Sepharose beads for each antibody were washed as described previously (Sutherland et al., 1988), resuspended in nonreducing sample buffer and frozen at −80°C until use. When all immunoprecipitations in the series were finished, the samples were run on 7% nonreducing SDS-polyacrylamide gels. The gels were fixed, treated with Enhance, dried and exposed to Kodak XAR-5 X-ray film.

Reverse transcription-polymerase chain reaction (RT-PCR) assays
Poly(A)+ RNA was isolated using the Micro-Fast Track kit (Invitrogen), from which single-stranded cDNA was synthesized using the cDNA Cycle kit (Invitrogen) and random hexamer primers. The cDNA was ethanol precipitated and resuspended in 30 µl of sterile water and 3 µl was used per 50 µl PCR reaction. The reaction mixture contained 0.01 M Tris (pH 8.3), 0.05 M KCl, 2.5 mM MgCl2, 0.1 mg/ml gelatin, 0.7 mM each of dATP, dGTP, dCTP and dTTP, 2.8 µM oligonucleotide primers and 0.5 U Taq 1 polymerase (AmpliTaq, Perkin-Elmer-Cetus Corp., Emeryville, CA). The primers used are listed in Table 1. For β1, α3, α5, α6 and α7 primers, PCR amplification was performed using a three-step process: (1) initial denaturation (4 minutes, 94°C); (2) five cycles of denaturation (94°C, 1 minute), annealing (65°C, 1 minute), and extension (72°C, 1 minute); and (3) 35 cycles of denaturation (94°C, 1 minute), annealing (60°C, 1 minute) and extension (72°C, 2 minutes). These steps were followed by a final extension (72°C, 15 minutes) and cooling to 4°C until use. For α1 and α2 primers, the process was identical except that annealing temperatures were lowered by 10°C. For α2, double amplification was performed; first the 2316-3540 primers were used, then 10% of each of these samples underwent the second amplification, in which the 3226-3453 primers were used. The amplified products were separated on 4% agarose gels using 1 kb DNA ladder (BRL) for size comparison. Primers for α2 were 5'-CCAGGACCTGCGACAATG-3' and 5'-CATCTTTGCGGACGAATG-3' and were the gift of Drs Barry Ziobek and Randall Kramer (Ziobek et al., in preparation). The 2316-3540 primers to α2 were the gift of Drs John Adelman and Clayton Buck. The identities of the amplified fragments were confirmed by restriction analysis, and controls for genomic DNA contamination included DNAse treatment of mRNA samples prior to reverse transcription, and PCR amplification of mRNA alone. Amplification was repeated on cDNA from at least three independent isolates of embryo mRNA to insure reliability of the data. Since variation was seen between individual cDNA isolates for a given primer pair, each new preparation of embryo cDNA was characterized using both β-actin primers (Rappolee et al., 1988) and integrin β1 primers to provide references for comparing results from other primer sets.

Unfertilized eggs and embryos at four different stages of preimplantation development were used: 2-cell, 8-cell, early blastocyst (32-40 cells, cavitation has just begun) and late blastocyst (hatched from the zona pellucida, with a layer of differentiated primitive endoderm on the inner cell mass). To examine integrin expression in postimplantation embryos, two samples were used: (1) embryo outgrowths, which contain differentiated trophoblast cells, primitive endoderm and primitive ectoderm, and (2) E7.5 embryos, which are at the mid- to late gastrula stage. Two regions of the E7.5 embryo were further isolated for mRNA preparation: the embryonic region (dissected at the level of the amnion) and the EPC. Trophoblast giant cells and decidual cells were removed from the EPC by manual dissection, leaving the core of undifferentiated trophoblast stem cells, which were then cultured in vitro for 4 days to obtain monolayers of differentiated trophoblast giant cells (Johnson and Rossant, 1981). Identical results were obtained in initial experiments using freshly dissected EPC (used without removing trophoblast giant cells) and cultured explants, so cultured explants were used in all further experiments in order to obtain specific results.

Table 1. Primers used for RT-PCR assays

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Forward primer</th>
<th>Sequence Location</th>
<th>Reverse primer</th>
<th>Sequence Location</th>
<th>Fragment size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1 (rat)</td>
<td>5’-CCCTGTAATGACCCAATGAGGAGG-3'</td>
<td>3407-3430</td>
<td>5’-GTCTGTATGTAAGTGATGTTTCCG-3'</td>
<td>3368-3711</td>
<td>252</td>
<td>Ignatius et al., 1990</td>
</tr>
<tr>
<td>α1 (human)</td>
<td>5’-TAATCCCTGCTTTGAAGCTTCC-3'</td>
<td>3226-3249</td>
<td>5’-TTAAGGCTATTCTGTTCGAGCCG-3'</td>
<td>3340-3453</td>
<td>227</td>
<td>Takada and Hemler, 1989</td>
</tr>
<tr>
<td>α1 (mouse)</td>
<td>5’-ATATGGCTCAGATCGACGAGG-3'</td>
<td>3223-3252</td>
<td>5’-GTACGCAGTATGAGCCG-3'</td>
<td>3526-3540</td>
<td>1225</td>
<td>Takada and Hemler, 1989</td>
</tr>
<tr>
<td>α2 (mouse)</td>
<td>5’-CTCGACGAGTCGTCGAGG-3'</td>
<td>880-903</td>
<td>5’-GAAGGCGGAGCTTGTGACGTACG-3'</td>
<td>1132-1155</td>
<td>275</td>
<td>Holers et al., 1989</td>
</tr>
<tr>
<td>α6 (mouse)</td>
<td>5’-TGGGAAACATATCC-3'</td>
<td>3032-3055</td>
<td>5’-GGAAATGCTGGTAGACGAGG-3'</td>
<td>3407-3430</td>
<td>398 (A)</td>
<td>Cooper et al., 1991</td>
</tr>
<tr>
<td>β1 (mouse)</td>
<td>5’-GTGACCATACGGCGGGAGGAGA-3'</td>
<td>2062-2086</td>
<td>5’-GTCGAAATATGCTTAAGGTTTCCA-3'</td>
<td>2253-2279</td>
<td>217</td>
<td>Holers et al., 1989</td>
</tr>
</tbody>
</table>

*Numbers correspond to those in the published hamster sequence (Tamura et al., 1991; Tsuji et al., 1990)
more material. Comparison of these two regions of the E7.5 embryo allowed us to determine relative expression of different integrin subunits in embryonic and extraembryonic tissues.

**Immunofluorescence**

Live embryos between the 2-cell and late morula stages were incubated 45 minutes at 37°C in medium containing primary antibody, rinsed three times, incubated 20 minutes at 37°C in medium containing secondary antibody, rinsed three times in medium and twice in PBS, then mounted on slides in PBS and viewed under epifluorescence. The zona pellucida gave high levels of background in live embryos at the 8-cell and later stages, so it was removed prior to staining by incubating the embryos in 0.002% chymotrypsin for 2 minutes, followed by two rinses in medium and manual removal. Embryos were allowed to recover for 4 hours prior to antibody incubation. Other methods of zona removal (pronase treatment, acid Tyrode’s buffer treatment) led to loss of staining.

Surface distribution of integrins on blastocysts was examined using fixed, rather than live, embryos as the amount of manipulation in the procedure resulted in a high rate of blastocoel collapse. Embryos were fixed for 15 minutes at room temperature in fresh 2% paraformaldehyde in PBS, rinsed twice in PBS, once in 0.15 M glycine in PBS, incubated 1 hour in PBS containing 0.1% BSA (PBS-BSA), and then in primary antibody diluted in PBS-BSA for 1 hour. Embryos were then rinsed three times in PBS-BSA, incubated for 30 minutes in rhodamine-conjugated secondary antibody diluted 1:100 in PBS-BSA, mounted on slides and viewed under epifluorescence. Parallel samples of early blastocysts were fixed in fresh 2% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS prior to staining to examine integrin distribution in inaccessible parts of the embryo. These embryos were viewed on a Nikon Microphot equipped with a confocal scanning laser (Biorad). Embryo outgrowths were grown on circular glass coverslips, fixed with cold methanol, rinsed with PBS and then stained as described previously (Sutherland et al., 1988).

**RESULTS**

**Integrin expression is regulated at the late blastocyst stage**

Integrin expression during the early development of the mouse embryo was examined using the reverse transcription-polymerase chain reaction (RT-PCR; Rappolee et al., 1988) to determine the mRNA profiles, and immunoprecipitation of metabolically labelled proteins to determine the corresponding protein profiles. We found two general patterns; continuous expression throughout the stages examined and regulated expression beginning at the time of implantation. Three integrin subunits were detected at both mRNA and protein levels throughout preimplantation development; mRNA for \( \alpha_6 \), \( \alpha_6 \) and \( \beta_1 \) was detectable in unfertilized eggs and in embryos of all preimplantation stages (Fig. 1), and sequential immunoprecipitation from lysates of \( ^{35} \)S-labeled embryos confirmed that the \( \alpha_6 \beta_1 \) and \( \alpha_6 \beta_3 \) heterodimers were expressed in preimplantation development. Another heterodimer, \( \alpha_6 \beta_3 \), was also detected in immunoprecipitates from all stages examined (4-cell, 8-cell, morula, early blastocyst, late blastocyst; Fig. 2).

In contrast, the expression of four other subunits was developmentally regulated. Messenger RNA for \( \alpha_6 \) was detectable at the 8-cell stage (Fig. 3), but the protein was only detectable in embryo outgrowths and cultured EPC (Fig. 2), suggesting that there may be posttranscriptional regulation of \( \alpha_6 \) expression. mRNA for \( \alpha_3 \) and \( \alpha_7 \) was first detected at the late blastocyst stage, at the time of endoderm segregation and just prior to implantation (Fig. 3). Both were expressed in embryo outgrowths and in E7.5 EPC explants (which consist of trophoblast derivatives), whereas only \( \alpha_2 \) was expressed in the E7.5 embryonic region, suggesting that \( \alpha_2 \) may be trophoblast-specific at this time. The \( \alpha_1 \) subunit was not detectable in preimplantation embryos, including the late blastocyst, at either the protein or the message level (Fig. 2), but \( \alpha_1 \) mRNA was present in embryo outgrowths after 24 hours of culture (Fig. 3), and is thus an early response to conditions that permit outgrowth. \( \alpha_1 \) was also expressed in the E7.5 embryonic region (Figs 2, 3), but was undetectable in the embryonic region (Fig. 3), suggesting that its expression, like that of \( \alpha_7 \), may be trophoblast-specific at this time.

The mRNAs for \( \alpha_6 \) and \( \alpha_3 \) each have two splicing variants that code for protein isoforms with different cytoplasmic domains (\( \alpha_6 \)A and B, and \( \alpha_3 \)A and B; Hogervorst et al., 1991; Tamura et al., 1991). To examine the developmental expression of these variants, we performed RT-PCR using primers which, in each case, bracket the segment spliced out of the B form (see Table 1 and references therein). The two forms of \( \alpha_6 \) mRNA give rise to amplifi-
cation products that differ in size by 130 bp, and the two forms of \( \alpha_3 \) mRNA to products that differ by 144 bp. We found that \( \alpha_6B \) was detectable in mRNA from unfertilized eggs and at all stages of preimplantation development (Fig. 4). In contrast, \( \alpha_6A \) was expressed at a relatively lower level in unfertilized eggs and variably in mRNA from 2-cell embryos, but was then undetectable until the late blastocyst stage (Fig. 4). Both variants were detectable in embryo outgrowths and E7.5 EPC explants, whereas the isolated E7.5 embryonic regions expressed only \( \alpha_6B \) (Fig. 4). Thus the expression of \( \alpha_6A \) is developmentally regulated and may be trophoblast-specific at E7.5. The presence of both variants in the unfertilized egg and 2-cell embryo probably represents maternal message, which normally undergoes degradation at the 2-cell stage (Flach et al., 1982; Clegg and Piko, 1983; Giebelhaus et al., 1983). In contrast to the pattern of splicing changes seen for \( \alpha_6 \), only \( \alpha_6A \) was expressed in either pre- or postimplantation embryos up to E7.5, as RT-PCR generated only the 660 bp fragment (Fig. 3).

These results show that integrin receptors for fibronectin (\( \alpha_5\beta_1 \)), laminin (\( \alpha_6\beta_1 \)) and vitronectin (\( \alpha_v\beta_3 \)) are expressed throughout preimplantation development, and are present long before the trophectoderm can attach and spread.

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**Fig. 2.** Immunoprecipitation of integrins from preimplantation embryos (A) and EPC explants (B) labelled with \([^{35}S]\)methionine/cysteine. (A) Immunoprecipitation from labelled lysates of preimplantation embryos using antibodies to the integrins \( \alpha_1 \) (lane 1), \( \alpha_2 \) (lane 2), \( \alpha_3 \) (lane 3), \( \alpha_5 \) (lane 4), \( \alpha_6 \) (lane 5), \( \alpha_5\beta_3 \) (lane 6) and \( \beta_1 \) (lane 7). The anti-\( \alpha_5\beta_3 \) results pictured here were from 8-cell embryos; all others were from late blastocysts. Results similar to those illustrated here were obtained for all preimplantation stages examined. (B) Immunoprecipitation from labelled lysates of EPC explants cultured 5 days, using antibodies to the integrins \( \alpha_1 \) (lane 1), \( \alpha_2 \) (lane 2), \( \alpha_3 \) (lane 3), \( \alpha_5 \) (lane 4), \( \alpha_6 \) (lane 5), and \( \alpha_5\beta_3 \) (lane 6). M, standards (\( \times10^{-3} \)) are indicated to the right of each panel, and the positions of the various subunits are indicated to the left.

**Fig. 3.** RT-PCR amplification of embryo cDNA showing developmentally regulated expression of \( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \) and \( \alpha_7 \) subunits. Amplification was performed on samples of 2-cell embryos (2C), 8-cell embryos (8C), early blastocysts (EB), late blastocysts (LB), embryo outgrowths (OG), ectoplacental cone explants (EPC), and isolated embryonic regions (E) of E7.5 embryos. The last lane (0) shows RT-PCR done without added cDNA. The results shown were obtained by using each set of primers on the same preparation of embryo cDNA, and \( \beta_1 \) primers were used as a positive control for each cDNA sample. Positions of DNA size markers are indicated to the right of the figure.
on any of these substrata. To determine whether redistribution of integrins might contribute to the acquisition of trophoblast adhesivity, we performed immunolocalization experiments using antibodies to α_6, α_5 and α_vβ_3. Staining for α_6 and α_vβ_3, but not α_5, was detected on the surface of the embryo from the 2-cell to the late morula/early blastocyst stage (32 cells) (Fig. 5). After blastocoel formation, only α_vβ_3 was detected on the surface of the embryo while both α_6β_1 and α_5β_1 were localized internally (Fig. 6). α_vβ_3 was present at the cell surface of all cells of the blastocyst, except the external surface of the trophectoderm (Fig. 6). The distribution of α_5β_1 at this stage was similar, although with a greater amount of intracellular staining (Fig. 6).

At the late blastocyst stage, surface staining for α_6, α_5 and α_vβ_3 gave the same results as in earlier blastocysts: staining for α_vβ_3 was detected on the external surface of the embryo, whereas no surface staining for either α_6 or α_5 was seen (Fig. 6). It is thus unlikely that redistribution of existing integrin receptors accounts for the onset of trophoblast adhesive behavior at the late blastocyst stage. These results also suggest that α_6β_1 and α_5β_1 may not mediate initial adhesion of trophoblast cells to laminin or fibronectin, respectively. In fact, both α_5 and α_6 were predominantly expressed in the inner cell mass in embryo outgrowths, and not in the spread trophoblast cells (Fig. 7). By contrast, α_vβ_3 was expressed in both the inner cell mass and in the spread trophoblast, and was localized to focal contacts in trophoblast cells (Fig. 7). Thus, of the integrin receptors that are expressed continuously during early development, only α_vβ_3 appears to be in a position to mediate primary trophoblast adhesion and migration.

In EPC explants, staining for α_6 was confined to the less differentiated cells in the center of the explant; no staining was seen in the trophoblast giant cells at the periphery of the explant (Fig. 7). This result suggests that α_6 expression by trophoblast is reduced with differentiation, explaining the paradox between the lack of detectable α_6 on trophoblast giant cells in embryo outgrowths and the presence of both α_6 mRNA and protein in EPC explants (Figs 1, 2). In contrast, α_5 and α_vβ_3 were detectable on the surface of all cells in the EPC explant, and α_vβ_3 was localized to focal adhesions in trophoblast giant cells (Fig. 7).

Molecular nature of trophoblast-laminin interactions

The results described above suggest that integrin-mediated trophoblast-laminin interactions are likely to involve newly synthesized receptors. All five of the integrin subunits showing regulated expression can form laminin receptors, and three appear to be trophoblast-specific at E7.5. Laminin...
Integrins in early mouse embryos

is a major component of the decidualized uterine stroma into which the trophoblasts invade; in fact, stromal fibroblasts express laminin only during implantation, while decreasing their production of fibronectin (Wewer et al., 1986; Glasser et al., 1987; Senior et al., 1988). We therefore examined trophoblast-laminin interactions more closely to determine which receptor(s) might be involved.

Outgrowth experiments were performed using laminin and laminin fragments (Fig. 8A) as substrata, in the presence or absence of antibodies to the laminin fragments. Embryos formed outgrowths on intact laminin and two of its fragments, P1′ and E8 (Fig. 8B). A small amount of attachment was seen to E1′ and to a recombinant form of the A chain COOH-terminal globular domain, which contains the putative binding site for the α3β1 integrin (Gehlsen et al., 1992). No trophoblast outgrowth was detected on either of these substrata, or on substrata of E3 or E4. Outgrowth on E8 and P1′ was specific, as assessed using substratum-specific antibodies (not shown).

To determine which binding sites are recognized in intact laminin, outgrowth assays were performed using heat-denatured laminin as a substratum. Heat treatment (80°C, 10 minutes) of intact laminin denatures the E8 binding site, leaving E1/P1 sites intact (Goodman et al., 1991). Embryos did not form outgrowths on heat-treated laminin (Δ-Ln; Fig. 8B), although a number of cultured cell types could attach and spread on this substratum (not shown). This suggests that only the E8 binding site is involved in trophoblast recognition of intact laminin, and that the P1′ binding site is cryptic.

There are two known integrin receptors for the P1′ domain, the α1β1 heterodimer, which mediates Arg-Gly-Asp (RGD)-independent interactions (Forsberg et al., 1990; Rossino et al., 1990) and the αvβ3 heterodimer, which mediates RGD-dependent interactions (Sonnenberg et al., 1990; Aumailley et al., 1991). Outgrowth on P1′ was inhibited by both GRGDSP peptides and polyclonal antiserum to αvβ3, but not by the control peptide GRGESP.
suggesting that $\alpha_v\beta_3$ is the major trophoblast P1′ receptor (Fig. 8C).

Trophoblast outgrowth on intact laminin and E8 was unaffected by mAb GoH3, which blocks $\alpha_6\beta_1$ interactions with laminin (Fig. 8C). Of the two known integrin receptors for the E8 region ($\alpha_6\beta_1$, Hall et al., 1990; Sonnenberg et al., 1990; and $\alpha_7\beta_1$, Kramer et al., 1991), $\alpha_6\beta_1$ is thus unlikely to be the sole or primary trophoblast integrin receptor for laminin. There are currently no antibodies that block the function of $\alpha_7\beta_1$ in the mouse, so the role of this integrin in trophoblast-laminin interactions could not be tested directly.

Another migratory cell population in the embryo, parietal endoderm cells, do appear to use $\alpha_6\beta_1$ as their sole or dominant laminin receptor. Embryo outgrowths cultured 4 to 5 days on fibronectin or laminin produced a population of small migratory cells that resembled parietal endoderm (PE) morphologically and expressed several PE markers (laminin, collagen type IV, cytokeratin and vimentin; Behrendtsen et al., 1992; data not shown), suggesting that they represent the in vitro equivalent of parietal endoderm. While trophoblast outgrowth on laminin was unaffected by the presence of mAb GoH3, PE cells aggregated on the periphery of the outgrowths and did not migrate further on the laminin substratum (Fig. 9). GoH3 did not affect PE migration on fibronectin substrata (Fig. 9). $\alpha_6\beta_1$ distribution in embryo outgrowths is consistent with these results; GoH3 stained the inner cell mass and migrating PE and not the trophoblast cells (Fig. 7). Thus $\alpha_6$ expression is decreased in differentiated trophoblast cells but remains prominent in parietal endoderm, where it is the primary receptor for adhesion and migration on laminin.

**DISCUSSION**

During the periimplantation development of the mouse embryo, cell-matrix interactions are likely to be particularly important for trophoblast differentiation and invasion during implantation. To analyze further the specific roles of integrin-mediated cell-matrix interactions in trophoblast invasion and in other early developmental events, we have characterized the patterns of expression of several integrin subunits and have investigated the role of individual integrins in the interactions between trophoblast and the extracellular matrix (ECM) component laminin.
A basic repertoire of integrins is expanded when extraembryonic lineages differentiate

Our results demonstrate that in the mouse, a basic repertoire of integrin heterodimers (α5β1, α6β1, α6β3) is expressed by the embryo throughout early development, whereas five other β1-associated α subunits (α1, α2, α3, α6A, α7) show developmentally regulated expression (Fig. 10). Thus the embryo always has the potential to interact with fibronectin (α5β1, α6β3), laminin (α6β1, α6β3) and vitronectin (α6β1) as well as with other αβ1 receptor ligands, notably thrombospondin. The later expression of additional α subunits allows greater diversity of cell-ECM interactions within the embryo and between the embryo and uterine tissues, by providing additional receptors for laminin (α1β1, α2β1, α5β1, α6Aβ1, α7β1) and fibronectin (α5β1) and collagens (α1β1, α2β1, α6β3).

The major point of regulation of integrin expression is the late blastocyst stage. There is little change in integrin expression during preimplantation stages, and none that correlates with any of the major developmental events of this period (compaction, blastocoel formation). α3 mRNA is detected during early preimplantation stages but the protein is detectable only in embryo outgrowths or postimplantation embryos. Expression of α2, α6A and α7 mRNA begins at the late blastocyst stage, before implantation, whereas α1 expression begins with embryo outgrowth or implantation (Fig. 10). Expression patterns in the E7.5 embryo suggest that at least three subunits (α1, α6A and α7) are specific to extraembryonic tissues, reflecting the fact that trophoblast and endoderm cells have the most extensive interactions with ECM at this time, and may therefore require the greatest diversity of receptors.

The variety of matrix receptors (e.g. integrins, cell surface galactosyltransferases and proteoglycans; Bayna et al., 1988; Sutherland et al., 1991) and ligands (fibronectin, laminin, collagen type IV, and thrombospondin; Wartiovaara et al., 1978; Cooper and MacQueen, 1981; O’Shea et al., 1990; Thorsteinsdottir, 1992) expressed in the preimplantation period begs the question of the role of cell-matrix interactions in early development. They are likely to be important for basement membrane formation on the inner surface of the trophectoderm and between the primitive ectoderm and primitive endoderm of the late blastocyst, but there is little evidence that integrin-mediated interactions are involved in other aspects of preimplantation development. Anti-integrin antiserum has no effect on preimplantation morphogenesis, whereas antibodies to the cell-cell adhesion molecule E-cadherin interfere with both compaction and primitive endoderm segregation (Damsky et al., 1983; Richa et al., 1985). Anti-integrin antiserum does block trophoblast outgrowth and parietal endoderm migration in vitro, suggesting that integrin-mediated cell-matrix interactions are critical to the function of extraembryonic cell types (Richa et al., 1985; Sutherland et al., 1988).

Integrin-mediated trophoblast-matrix interactions may involve newly expressed receptors

Previous results have shown that integrin receptors play an important role in trophoblast outgrowth on fibronectin, laminin and collagen type IV, as a pan-integrin antibody will completely block outgrowth on these substrata (Sutherland...
...et al., 1988). Of the integrin receptors expressed during preimplantation stages, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ are both localized to the interior of the embryo at the late blastocyst stage, while $\alpha_5\beta_3$ is also on the external trophoderm surface at this time. As $\alpha_6\beta_3$ can recognize many, but not all, of the substrata on which embryos can form outgrowths, the ability of trophoblast cells to interact with ECM components may be acquired as a result of either a matrix contact-induced redistribution of the existing receptors, the availability of new receptors, or both.

Trophoblast outgrowth on laminin is likely to involve newly expressed integrin receptors, as neither $\alpha_6\beta_1$ nor $\alpha_5\beta_3$, laminin receptors expressed prior to implantation, appeared to play a role in trophoblast outgrowth on intact laminin substrata. Results described here (Fig. 8), and by Armant (1991), show that trophoblast cells interact with intact laminin via site(s) in the E8 region. Of the two known integrin receptors for the E8 region ($\alpha_6\beta_1$, $\alpha_7\beta_1$), we were able to rule out $\alpha_6\beta_1$ as a likely trophoblast laminin receptor because outgrowth on laminin and E8 was not perturbed by the function-blocking anti-$\alpha_6$ antibody GoH3. In addition, expression of $\alpha_6$ appeared to be downregulated in migrating trophoblast giant cells (Fig. 7). The $\alpha_7\beta_1$ complex is thus the most likely candidate to mediate integrin-dependent trophoblast-laminin interactions. Other evidence supports the role of $\alpha_7\beta_1$ as a trophoblast laminin receptor: mRNA for $\alpha_7$ is first expressed just before implantation and it appears to be trophoblast-specific in the early postimplantation period. Our data suggest that trophoblast and endoderm lineages use different strategies to interact with laminin, as

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**Fig. 9.** Embryo outgrowths on laminin and fibronectin in the presence of function-perturbing antibodies to integrin $\alpha_6$. Embryos were cultured on laminin (A,B) and fibronectin (C,D) in the presence of normal rabbit serum (Control; A,C) or of mAb GoH3 (Anti-$\alpha_6$ (GoH3); B,D). Trophoblast cells are indicated by arrowheads, and the smaller endoderm cells by asterisks. Scale bar, 25 $\mu$m.

**Fig. 10.** Diagram summarizing the pattern of integrin subunit expression seen in perimplantation mouse embryos.
parietal endoderm cell migration on laminin was inhibited by GoH3, indicating that α3β1 is a major laminin receptor for these cells. Other non-integrin E8 receptors could also be involved in trophoblast-laminin interactions. Cell-surface galactosyltransferase has been shown to play a role in secondary giant cell outgrowth from E8 explants (Romagnano and Babiarz, 1990), but it may not have the same function in primary giant cell migration from embryo outgrowths (Arman, 1991). Non-integrin proteins that recognize a laminin A chain region containing the amino acid sequence IKVAV (reviewed by Mecham, 1991) could also play a role; there is currently no information about their expression or function in early mouse embryos.

Trophoblast cells can also recognize the P1′ region when it is presented as a fragment. The cryptic nature of this interaction is consistent with previous results documenting a cryptic RGD-dependent cell binding site in the cross region of laminin which is exposed by proteolysis (Nurcombe et al., 1989; Aumailley et al., 1991). Laminin and the P1′ fragment have been shown to have distinct effects on cell behavior: laminin promotes, but P1′ inhibits, melanoma metastasis to the lung (Terrarova et al., 1984), and P1′ more effectively stimulates collagenase activity (Turpeenniemi-Hujanen et al., 1986; Sang et al., 1991). The significance of trophoblast interactions with the cryptic P1′ site is unknown, but a role for such interactions in implantation cannot be ruled out. Trophoblast invasion is accompanied by considerable matrix degradation (Glass et al., 1983; Behrendtsen et al., 1992), which may result in the production of laminin fragments with RGD-dependent cell binding activity.

There are two known integrin receptors for the P1′ fragment of laminin; the α3β1 heterodimer (Forsberg et al., 1990; Rossino et al., 1990; Kramer et al., 1991) and the α6β1 heterodimer (Sonnenberg et al., 1990). Our data indicate that α3β1 is the major trophoblast P1′ adhesion receptor, as outgrowth on this fragment was blocked by RGD-containing peptides and antibodies against the α3β1 complex. Whether α3β1 mediates any trophoblast-laminin interactions could not be tested directly, as no suitable antibodies are available. However, the pattern of α3 expression suggests that this integrin may play an important role in trophoblast function during implantation; its expression is trophoblast specific at early postimplantation stages, and the late onset of its expression suggests that it is upregulated in response to trophoblast-matrix interactions. Studies by Calof and Lander (1991) suggest that, in neurons, interaction with the P1′ region of laminin may promote motility rather than adhesion, so it is possible that α3β1-laminin interactions in trophoblasts transduce signals that are not primarily adhesive.

Expression of α3β1 is also developmentally regulated during human trophoblast differentiation. It is expressed by differentiated trophoblast cells that have invaded the uterine wall, but not by stem cells or partially differentiated trophoblasts (Damsky et al., 1992). Expression of α3β1 is induced in an in vitro model that mimics human trophoblast differentiation and invasion, and function-blocking antibodies against the α3 subunit can greatly reduce human trophoblast invasion through a reconstituted basement membrane (Librach et al., 1991; Damsky et al., in preparation). Taken together, the data from the mouse and human systems suggest that the α3β1 may play an important role in trophoblast invasion.

In summary, we have found that the expression of integrin receptors is developmentally regulated in mouse embryos, with a basic repertoire that is continuously expressed and additional receptors that are expressed when extraembryonic lineages differentiate. We have identified two major sites of adhesive interactions between trophoblast and laminin (E8 and P1′) and the integrin receptor for the P1′ site (α3β1). However, it is likely that integrin-mediated interactions with laminin and with other ECM components will have effects on trophoblast behavior and function beyond those involved in attachment and migration uncovered thus far. The use of in vitro culture models of trophoblast invasion in conjunction with antisense, antibody or gene-targeting approaches should enable direct testing of these ideas in the future.

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