Effect of 32/67 kDa laminin-binding protein antibody on mouse embryo implantation

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Mouse embryo implantation depends on the complex interaction between the embryo trophoblast cells and the uterine environment, which deposits an extracellular matrix with abundant amounts of laminin. Intrauterine injection and blastocyst or ectoplacental cone culture models were used to study the effect of 32/67 kDa laminin-binding protein antibody on mouse embryo implantation *in vivo* and *in vitro*. Intrauterine injection of 32/67 kDa laminin-binding protein antibody (0.4 mg in 1 ml Ham's F-10 medium, 5 µl per mouse) into the left uterine horns of mice ($n = 22$) on day 3 of pregnancy inhibited embryo implantation significantly ($P < 0.001$) compared with the contralateral horns that had been injected with normal rabbit IgG. A continuous section study on day 5 after injection showed that the embryos in the control uteri implanted normally and developed healthily, but there were no embryos or the remaining embryos had disintegrated in the uteri injected with 32/67 kDa laminin-binding protein antibody. Blastocysts or ectoplacental cones were cultured in media containing 32/67 kDa laminin-binding protein antibody (0.2 mg ml⁻¹) on laminin-coated dishes with normal rabbit IgG at the same concentration as in the controls. The 32/67 kDa laminin-binding protein had no effect on blastocyst or ectoplacental cone attachment, but prohibited the blastocyst or ectoplacental cone outgrowth and primary or secondary trophoblast giant cell migration. These results indicate that 32/67 kDa laminin-binding protein antibody blocked mouse embryo implantation by preventing embryo trophoblast cell invasion and migration through the uterine decidual basement membrane-like extracellular matrix which has a high laminin content.

Introduction

Mouse embryo implantation depends on the interactions between the trophoblast cells and the surrounding uterine environment. During normal development, the initial invasion by primary trophoblast stimulates the differentiation of the uterine stromal fibroblasts to decidua. The stromal cells increase in size and deposit a pericellular basement membrane-like matrix consisting of laminin, fibronectin, and collagen type IV (Wewer *et al.*, 1986). The secondary trophoblast, derived from the ectoplacental cone (EPC), invades the decidua and forms contacts with the maternal blood supply that lead to the establishment of a haemochorial placenta (Schlafke and Enders, 1975; Romagnano and Babiartz, 1993).

Conversion of the epithelial trophectoderm to invasive trophoblast begins at the late blastocyst stage, and comprises a change in adhesive behaviour and the onset of motility. The first signs of embryo outgrowth are detected about 10–15 h

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.*, 1993).

Attachment and outgrowth of both primary (Armant *et al.*, 1986) and secondary (Romagnano and Babiartz, 1990) trophoblast cells are supported by laminin *in vitro*. Laminin is a multidomain glycoprotein containing active sites that promote the adhesion and migration of many cell types, including metastatic cells (Beck *et al.*, 1990). YIGSR (Tyr-Ile-Gly-Ser-Asp) located on the B₁ chain (Graf *et al.*, 1987), IKVAV (Ile-Lys-Val-Ala-Val) on the long arm (Tashiro *et al.*, 1989) and RGD (Arg-Gly-Asp) on the short arm (Grant *et al.*, 1989) of the A chain are the main functional peptide sequences of laminin. The YIGSR peptide sequence is recognized by a 67 kDa laminin-binding protein (LBP) (Graf *et al.*, 1987), while IKVAV is recognized by laminin-binding proteins of 32, 45, 67 and 80 kDa (Clement *et al.*, 1990), and there is an immunological relationship among these proteins. The cDNA clone of the 67 kDa laminin-binding protein predicts a 32 kDa protein (Wewer *et al.*, 1986 and Yow *et al.*, 1988), and antiserum to the 32 kDa protein recognizes the 32,

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45 and 67 kDa laminin-binding proteins (Mercurio and Shaw, 1988; Thompson *et al.*, 1989). The RGD sequence is recognized by the integrin family of receptors, such as $\alpha_1\beta_1$ and $\alpha_v\beta_3$, and the developmental regulation of integrin expression appears to have functional significance for trophoblast invasion of the laminin-rich uterine stroma (Sutherland *et al.*, 1993). The 32/67 kDa LBPs are laminin non-integrin receptors and express on trophoblast cell surfaces (Romagnano and Babiarz, 1993). The present study investigated the effect of 32/67 kDa LBP antibody on mouse embryo implantation using an *in vivo* and *in vitro* implantation model. This antibody was rabbit serum made from a 17-amino-acid peptide from the N-terminus (residues 25-41) of the mouse 67 kDa LBP cDNA clone encoding a 32 kDa protein (Clement *et al.*, 1990), which forms a 67 kDa mature protein by dimerization (Landoski *et al.*, 1995).

Materials and Methods

Materials

Mouse laminin from Englebreth-Holm-Swarm (EHS) tumour basement membrane was obtained from Sigma Chemical Co. (St. Louis, MO). Ham's F-10 nutrient mixture and fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, NY). Rabbit anti-32/67 kDa IgG was a gift from H. Kleinman (National Institute of Dental Research, NIH, Bethesda, MD).

Animals

Young adult female mice (25–30 g) of the Kunming white strain were obtained from the Feed Lot of the Zoo, Institute of Zoology, Chinese Academy of Sciences (Beijing) and maintained at 22–25°C on a 14 h light:10 h dark cycle. Female mice were mated naturally with 12-week-old males of the same strain (one female:one male in a cage overnight) for the intraluminal injection of antibody experiment. Other females were superovulated by injecting i.p. 7.5 iu eCG (Institute of Changchun Biological product, Department of Health, China), and then, approximately 48 h after eCG injection, these females were injected i.p. with 7.5 iu hCG (Institute of Zoology, Chinese Academy of Sciences, China) and then impregnated in a similar way. Mating was evidenced by the appearance of a vaginal plug the next morning. The day of the appearance of a vaginal plug was designated as day 1 of pregnancy and from this time, animals were caged separately.

Intrauterine injection

Naturally ovulated animals were prepared as described by Zhu *et al.* (1995) on day 3 of pregnancy. In the experimental group of 22 mice, the left uteri were injected with 5 μ l 32/67 kDa LBP IgG (0.4 mg ml⁻¹ in Ham's F-10 medium) by syringe via the uterine horns, where the oviduct connects with uterus. All the right uteri were injected with

5 μ l normal rabbit IgG at the same concentration in Ham's F-10 medium as in the control group. The animals appeared healthy throughout the experiment and were killed by CO₂ inhalation on day 5 after injection, and the numbers of implanted embryos were counted under a dissecting microscope.

Morphological analysis

Embryo implantation in the uteri of rabbits injected with normal rabbit IgG was compared with that in uteri treated with 32/67 kDa LBP IgG. On day 5 after intrauterine injection, the right and left uteri were removed and fixed in 10% (v/v) formalin in PBS for haematoxylin and eosin staining.

Blastocyst collection and culture

Blastocysts were flushed from the uteri on day 4 of pregnancy with uterus flush medium (UFM: serum-free Ham's F-10 supplemented with 1.6 mg NaHCO₃ ml⁻¹, 0.3 mg L-glutamine ml⁻¹, 0.24 mg calcium lactate ml⁻¹ and 400 U *Gentamycini sulfatis* ml⁻¹; Gibco BRL, Grand Island, NY). After three washes with UFM, these blastocysts were transferred to microdrops (50 μ l) under mineral oil on 35 mm tissue culture dishes (Falcon 1008; Becton-Dickinson, Lincoln Park, NJ). The blastocysts were cultured in a 5% CO₂ humidified air incubator at 37°C with blastocyst hatching medium (UFM supplemented with 4 mg BSA ml⁻¹ and 3.67×10^{-6} mol oestradiol l⁻¹; Sigma, St Louis, MO) for 24 h (Zeng and Cao, 1996). The blastocysts hatched from the zona pellucida were collected and transferred randomly to blastocyst culture medium (UFM supplemented with 1% heat-inactivated FCS) containing 32/67 kDa LBP IgG (0.2 mg ml⁻¹) or normal rabbit IgG at the same concentration and cultured for 48 h. The culture dishes were pre-coated at room temperature for 3 h under a clean air hood with 5 μ l laminin (1 mg ml⁻¹).

Isolation and culture of ectoplacental cone

Female mice were killed on day 8 of pregnancy and uteri were removed to Hank's balanced salt solutions (HBSS) for dissection. Decidual capsules were removed from the uterus and split open with fine forceps. The embryo and attached trophoblast were separated from the surrounding decidua. EPCs were dissected at the junction with the extra-embryonic ectoderm by fine needles and moved to culture dishes using pipettes. Any maternal blood surrounding the EPC was removed by washing six times with HBSS. EPCs were cultured in Ham's F-10 medium containing 4% heat-inactivated FCS, which was optimal for EPC growth (Zhang *et al.*, 1995), with 32/67 kDa LBP IgG (0.2 mg ml⁻¹) or normal rabbit IgG at the same concentration for 48 h. The 24-well dishes were pre-coated at room temperature for 3 h in the air clean bench with 10 μ l laminin (1 mg ml⁻¹).

Data collection and statistical analysis

Implanted embryos were identified by the presence of a decidual capsule. Blastocyst or EPC attachment and outgrowth percentages were recorded under an Olympus inverted phase-contrast microscope at 48 h. The migrating distance of primary (PTGC) or secondary trophoblast giant cells (STGC) from the inner cell mass (ICM) or EPC rudiment was measured by eyepiece grid. The quantitative data are given as means $\pm$ SEM and were analysed by Student's *t* test for paired comparisons (*in vivo* results) or ANOVA with Scheffé's test (*in vitro* results). Differences were considered significant at $P < 0.05$.

Results

Effect of 32/67 kDa laminin-binding protein IgG intrauterine injection on mouse embryo implantation

The animals appeared healthy throughout the experiment and were killed on day 5 after the intrauterine injection. In 22

naturally ovulated mice, the mean number of implanted embryos for each uterus injected with 32/67 kDa LBP IgG was 2.17 ± 1.04 ($n = 48$), compared with 6.19 ± 1.22 ($n = 136$) in each contralateral uterus injected with normal rabbit IgG. This difference was significant ($P < 0.001$), indicating that administration of 32/67 kDa LBP IgG blocked mouse embryo implantation.

Morphological analysis of intrauterine-injected uteri

During normal mouse embryo implantation, the embryo attached to and invaded the uterine epithelium on day 5 of pregnancy (Fig. 1a,b). The serial sections on the day 5 after intrauterine injection (day 8 of pregnancy) showed that embryos in control uteri implanted normally and developed healthily (Fig. 1c). However, there were no embryos or the remaining embryos were abnormal and had disintegrated in the 32/67 kDa LBP IgG injected uteri (Fig. 1d). The remaining embryos were usually localized at the bottom of the uterus, away from the uterine horn where the antibody was injected.

Fig. 1. Morphology of mouse embryo implantation (paraffin wax sections stained with haematoxylin and eosin). Uteri injected with normal rabbit IgG (a–c) compared with uteri injected with 32/67 kDa laminin-binding protein IgG-treated uteri (d). (a,b) The process of mouse embryo attachment and invasion to the uterine epithelium on day 5 of pregnancy. (c,d) Implantation site sections of the identical mouse right and left uteri on day 5 after intrauterine injection. The embryo in (c) implanted successfully and developed normally, while the remaining embryo in the 32/67 kDa LBP antibody-injected uterus (d) degenerated and disintegrated. BL, blastocyst; Em, embryo; UE, uterine epithelium. Scale bars represent 40 μ m.

Blastocyst and ectoplacental cone culture assays

The hatched blastocysts were cultured on laminin (Fig. 2a). Laminin promoted blastocyst outgrowth, which consisted of a monolayer of spreading trophoblast cells with the ICM remaining on top in the medium containing normal rabbit IgG (Figs 2b and 3). Blastocyst outgrowth and PTGC migration were inhibited in the medium containing 32/67 kDa LBP IgG (Figs 2c and 3). Laminin promoted EPC outgrowth, which consisted of a monolayer of migrating STGC with the EPC remaining on top in the medium containing normal rabbit IgG (Fig. 2d). EPC outgrowth or STGC migration from the EPC rudiment was inhibited in the medium containing 32/67 kDa LBP IgG (Figs 2e and 3). However, the presence of 32/67 kDa LBP IgG did not affect blastocyst or EPC attachment, although it did inhibit EPC attachment slightly (Fig. 3).

Discussion

Cell-matrix interactions are likely to be particularly important for trophoblast differentiation and invasion during implantation. Laminin is a major component of the decidualized uterine stroma which the trophoblast invades,

and this matrix protein may influence trophoblast migration since its expression is upregulated at the time of implantation (Burrows *et al.*, 1996).

Laminin has multiple cell-binding sites, such as the RGD of the A chain short arm, the YIGSR of the B₁ chain and the SIKVAV of the A chain long arm, which are recognized by various cell surface receptors. For example, RGD is recognized by members of the integrin superfamily (Sutherland *et al.*, 1993). YIGSR or SIKVAV may be potential laminin-binding sites for the 67 kDa laminin-binding protein (Romagnano and Babiarz, 1993).

A 67 kDa protein has been identified as a laminin receptor in tumour and muscle cells, and Wewer *et al.* (1986) reported a partial cDNA encoding the human 67 kDa protein. Several groups (for example, Yow *et al.*, 1988) have isolated human and mouse full-length cDNA, the deduced amino acids sequences of which match the cDNA clone described by Wewer *et al.* (1986). However, these full-length cDNA clones code a 32 but not a 67 kDa protein. The 32 kDa protein has been identified as a cell-surface, laminin-binding protein in various cells, and antibodies to a bacterial fusion protein from a full-length clone for the 32 kDa protein recognize the 32, 45 and 67 kDa proteins. These proteins may be encoded by distinct genes while sharing a common epitope (Clement *et al.* 1990).

Fig. 2. Mouse blastocyst or ectoplacental cone (EPC) culture on laminin. (a) Hatched blastocyst. (b) Blastocyst outgrowth and migration of primary trophoblast giant cells (PTGCs) in medium containing normal rabbit IgG (0.2 mg ml^{-1}). (c) Inhibition of blastocyst outgrowth and PTGC migration in the presence of 32/67 kDa laminin-binding protein (LBP) IgG (0.2 mg ml^{-1}). (d) EPC outgrowth and migration of secondary trophoblast giant cells (STGCs) in medium containing normal rabbit IgG (0.2 mg ml^{-1}). (e) Inhibition of EPC outgrowth and STGC migration in the presence of 32/67 kDa LBP IgG (0.2 mg ml^{-1}). BL, blastocyst; ICM, inner cell mass. Scale bars represent $50 \mu\text{m}$.

Fig. 3. Effect of 32/67 kDa laminin-binding protein (LBP) IgG to mouse blastocyst or ectoplacental cone (EPC) attachment, outgrowth and migration of primary (PTGCs) or secondary trophoblast giant cells (STGCs) on laminin. 32/67 kDa LBP IgG did not affect blastocyst (BL) or EPC attachment, and inhibited blastocyst and EPC outgrowth and PTGC or STGC migration. □, Normal rabbit IgG (0.2 mg l⁻¹); ■, 32/67 kDa LBP IgG (0.2 mg l⁻¹). There were at least 25 blastocysts or EPCs in each group and the experiment was repeated three times. Asterisks represent significant differences: ***P* < 0.01; ****P* < 0.001.

The 32/67 kDa LBP IgG was made with a peptide of 17 amino acids from the NH₂ end of the 67 kDa laminin-binding protein, the cDNA of which encodes a 32 kDa protein. This antibody recognizes several laminin-binding proteins, including the 32 and 45 kDa proteins (Clement *et al.*, 1990). Several crossreacting proteins were found on mouse trophoblast (33, 41, 45, 62 and 76 kDa) (Romagnano and Babiarz, 1993). The antiserum also identifies IKVAV binding proteins isolated by affinity chromatography (Clement *et al.*, 1990). Further details of the structure or binding specificities of these molecules remain controversial (Mecham, 1991).

A role for these proteins in trophoblast-laminin interaction is demonstrated by the inhibition of blastocyst or EPC outgrowth and PTGC or STGC migration in the presence of the antibody. The binding of 32/67 kDa LBP IgG may not affect the ability of trophoblast to bind to laminin initially, but subsequent blastocyst or EPC outgrowth and PTGC or STGC migration may be inhibited by a decrease in protein mobility owing to antibody cross-linking of the laminin-binding proteins. The 32/67 kDa LBP IgG did not affect blastocyst or EPC attachment, which is consistent with the finding that the putative peptide substrate cyclic YIGSR had no effect on blastocyst attachment (Y. Cao, C. Zhang, E. Duan, G. Jiang and G. Zeng, unpublished), but inconsistent with an earlier EPC attachment experiment, in which cyclic YIGSR prevented EPC attachment on laminin in a dose-dependent manner (Zhang *et al.*, 1996). Moreover, the attachment on laminin was also inhibited by incubating EPC with another putative peptide, IKVAV (Romagnano and Babiarz, 1993). The LBPs on secondary trophoblast from EPC may interact with different, uncharacterized active sites on laminin. The

epitope of the 32/67 kDa LBP antibody remains to be identified in order to resolve this puzzle.

The present study showed that 32/67 kDa LBP IgG had no effect on blastocyst or EPC attachment, but prevented blastocyst or EPC outgrowth and primary or secondary trophoblast cell migration on laminin *in vitro*, and blocked embryo implantation *in vivo*. The results indicated that this antibody blocks implantation by preventing embryo trophoblastic cell invasion and migration through the uterine decidual basement membrane-like extracellular matrix, which is rich in laminin. Monkeys showed fetal loss after immunization with YIGSR (Chambers *et al.*, 1995), which is a further demonstration that the interaction between LBPs and laminin is important for reproductive processes. A cell-laminin interaction via a 67 kDa LBP has been shown to be an important step in the signal transduction pathway (Gloe *et al.*, 1999). Incubation of neuroblastoma cells with C(YIGSR)₃-NH₂ peptide amide or antibody directed against the 67 kDa LBP induces tyrosine phosphorylation of proteins (Bushkin-Harav and Littauer, 1998). Further studies on the mechanism of signalling among the trophoblast cell surface, 32/67 kDa LBP(s) and uterine decidual laminin are needed to increase understanding of the process of embryo implantation.

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References

- Armant DR, Kaplan HA and Lennarz WJ (1986) Fibronectin and laminin promote *in vitro* attachment and outgrowth of mouse blastocysts *Developmental Biology* **116** 519–523
- Beck K, Hunter I and Engel J (1990) Structure and function of laminin: anatomy of a multidomain glycoprotein *FASEB Journal* **4** 148–160
- Burrows TD, King A, and Loke YW (1996) Trophoblast migration during human placental implantation *Human Reproduction Update* **2** 307–321
- Bushkin-Harav I and Littauer UZ (1998) Involvement of the YIGSR sequence of laminin in protein tyrosine phosphorylation *FEBS Letters* **424** 243–247
- Chambers BJ, Klein NW, Conrad SH, Ruppenthal GC, Sackett GP, Weeks BS and Kleinman HK (1995) Reproduction and sera embryotoxicity after immunization of monkeys with the laminin peptides YIGSR, RGD, and IKVAV *Proceedings National Academy of Sciences USA* **92** 6818–6822
- Clement B, Segul-Real B, Savagner P, Kleinman HK and Yamada Y (1990) Hepatocyte attachment to laminin is mediated through multiple receptors *Journal of Cell Biology* **110** 185–192
- Gloe T, Riedmayr S, Sohn H-Y and Pohl U (1999) The 67 kDa laminin-binding protein is involved in shear stress-dependent endothelial nitric-oxide synthase expression *Journal of Biological Chemistry* **274** 15 996–16 002
- Graf J, Ogle RC, Robey FA, Sasaki M, Martin GR, Yamada Y and Kleinman HK (1987) A pentapeptide from the laminin B1 chain mediates cell adhesion and binds the 67 000 laminin receptor *Biochemistry* **226** 6896–6900
- Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR and Kleinman HK (1989) Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures *in vitro*. *Cell* **58** 933–943
- Landowski TH, Dratz EA and Starkey JR (1995) Studies of the structure of the metastasis-associated 67 kDa laminin-binding protein: fatty acid acylation and evidence supporting dimerization of the 32 kDa gene product to form the mature protein *Biochemistry* **34** 11 276–11 287
- Mercurio AM and Shaw LM (1988) Macrophage interaction with laminin: PMA selectively induces the adherence and spreading of mouse macrophages on a laminin substratum *Journal of Cell Biology* **107** 1873–1880
- Mecham RP (1991) Receptors for laminin on mammalian cells *FASEB Journal* **5** 2538–2546
- Romagnano L and Babiarz B (1993) Mechanisms of murine trophoblast interaction with laminin *Biology of Reproduction* **49** 374–380
- Schlafke S and Enders AC (1975) Cellular basis of interaction between trophoblast and uterus at implantation *Biology of Reproduction* **12** 41–65
- Sutherland AE, Calarco PG and Damsky CH (1993) Developmental regulation of integrin expression at the time of implantation in the mouse embryo *Development* **119** 1175–1186
- Tashiro K, Sephel G C, Grotorex D, Sasaki M, Shirashi N, Martin GR, Kleinman HK and Yamada Y (1989) A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration and neurite outgrowth *Journal of Biological Chemistry* **264** 16 174–16 182
- Thompson HL, Burbelo PD, Segul-Real B, Yamada Y and Metcalfe DD (1989) Laminin promotes mast cell attachment *Journal of Immunology* **143** 2323–2327
- Wewer UM, Damjanov A, Weiss J, Liotta IA and Damjanov I (1986) Mouse endometrial stromal cells produce basement-membrane components *Differentiation* **32** 49–58
- Yow H, Wong JM, Chen HS, Lee C, Steele GD and Chen LB (1988) Increased mRNA expression of laminin-binding protein in human colon carcinoma: complete sequence of a full-length cDNA encoding the protein *Proceedings National Academy of Sciences USA* **85** 6394–6398
- Zeng GQ and Cao YJ (1996) Co-culture of mouse embryos with uterine epithelial cells monolayer *Acta Zoologica Sinica* **42** 212–217
- Zhang CY, Cao YJ and Zeng GQ (1995) The model of murine ectoplacental cone implantation *in vitro*. *Journal of Reproductive Medicine* **4** 173–174
- Zhang CY, Cao YJ and Zeng GQ (1996) Studies of murine ectoplacental cone cells interaction with laminin *Acta Biologica Experimentalis Sinica* **29** 125–131
- Zhu ZM, Kojima N, Stroud MR, Hakomori S, and Fenderson BA (1995) Monoclonal antibody directed to Le^x oligosaccharide inhibits implantation in the mouse *Biology of Reproduction* **52** 903–912