Expression and localization of $\alpha_v\beta_6$ integrin in extraplacental fetal membranes: possible role in human parturition

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Successful outcome of human parturition is dependent upon extensive remodelling of the extracellular matrix (ECM) of the cervix, uterus and fetal membranes, a process that involves adhesion molecules and is also common in tumour invasion and metastasis. To elucidate the role of integrins in human parturition, this study characterizes the expression of the tumour-associated $\alpha_v\beta_6$ integrin in human placenta and extraplacental membranes. Immunohistochemical analysis of the placenta and fetal membranes from normal vaginal deliveries (NVD) ($n = 10$) exhibited strong intensity of staining for $\alpha_v\beta_6$ integrin (3 = dark brown) in the epithelial layer of the amnion. Weak immunohistochemical staining of $\alpha_v\beta_6$ integrin (1 = pale brown) was detected in the chorion and at the decidual edge. These results were consistent with the immunodetection of $\alpha_v\beta_6$ integrin by western blot analysis that showed 4-fold enhanced expression in the amnion compared to chorion of both NVD and term elective caesarean section (CS) deliveries. Even though there was no difference in the extent of immunohistochemical staining of $\alpha_v\beta_6$ integrin between the amnion of NVD and CS groups, significantly higher intensity of staining was observed in the NVD amniotic epithelium compared to that of CS ($n = 10$) ($\chi^2 = 10.25, P = 0.0059$). Western blot analysis of the fetal membranes showed no differences in the expression of $\alpha_v\beta_6$ integrin between the NVD and CS groups. Gelatin zymography demonstrated the presence of pro-matrix metalloprotein-9 (MMP-9) and pro-MMP-2 in the amnion and chorion of NVD, whereas in CS only the presence of pro-MMP-2 was observed. These results suggest that in term pregnancy, human fetal membranes express $\alpha_v\beta_6$ integrin and that the expression is significantly higher in amnion compared to chorion. The fact that enhanced expression of $\alpha_v\beta_6$ integrin in fetal membranes correlates with the expression of pro-MMP-9 in NVD is consistent with the invasive role of the integrin in cancer and suggests that the molecule may have a proteolytic role in the initiation and progression of labour.

Key words: amnion/chorion/integrin/parturition/placenta

Introduction

Of all the human tissues, placenta is unique in its ability to proliferate and invade another tissue in a controlled fashion and is thus a useful model for the study of an invasive mechanism under normal physiological conditions and to differentiate that from the processes involved in tumour progression. Over the past decade, insight into the mechanisms regulating cell adhesion, migration and invasion has been linked to cell surface molecules known as integrins (Hynes, 1992). The integrins are a group of transmembrane glycoproteins consisting of $\alpha$ and $\beta$ subunits that are integrated across the plasma membrane to provide a link between the extracellular matrix (ECM) and the cytoskeletal molecules within the cells. There are two major families of integrins: those in which the $\beta_1$ chain combines with any one of nine $\alpha$ chains, and the $\alpha_v$ group in which the $\alpha_v$ associates with either $\beta_1$, $\beta_3$, $\beta_5$ or $\beta_8$ subunits. The variable units in both groups determine the ECM ligand specificity (e.g. fibronectin, laminin, vitronectin or tenasin) for individual receptors.

$\alpha_v\beta_6$ integrin has been shown to be highly expressed in developing fetal tissues and in a number of epithelial carcinomas and cancer cell lines (Breuss et al., 1993). In adult tissues, $\alpha_v\beta_6$ integrin plays a role in inflammation and wound healing through the facilitation of the migration of epithelial cells to traumatized areas and in the re-establishment of an intact epithelium at injured sites (Haapasalmi et al., 1996). The expression of $\alpha_v\beta_6$ integrin has been demonstrated in colonic and pancreatic carcinomas while no such expression was found in normal epithelium of the same origin (Weinacker et al., 1994). Increased expression of $\alpha_v\beta_6$ integrin has been reported in a range of epithelial neoplasms (Jones et al., 1997; Arihiro et al., 2000; Ahmed et al., 2002c) and in pre-malignant oral epithelial dysplasias (Hamidi et al., 2000) suggesting that this integrin may be involved in malignant transformation. Induced or endogenous expression of $\alpha_v\beta_6$ integrin in colon, ovarian and oral squamous carcinoma cells has been reported to promote proliferation (Agrez et al., 1994), enhance matrix metalloproteinase-9 (MMP-9) secretion (Agrez et al., 1999; Thomas
et al., 2001b; Ahmed et al., 2002b) and increased matrix degradation (Thomas et al., 2001a; Ahmed et al., 2002a). These data suggest that $\alpha_\beta_3$ integrin may initiate an invasive phenotype in transformed cells partially through alterations in the cellular proteolytic activity mediated by MMP-9.

Although integrins have been implicated in placentation, particularly in the migration and invasion of intermediate (extravillous) cytotrophoblasts (CTB), the involvement of integrins in labour and delivery has not been investigated. During early pregnancy, CTB infiltrate the basal plate and invade the uterine spiral arteries, replacing the endothelial lining of these vessels. This process is requisite for establishing the flow of maternal oxygenated blood to the fetus (Zhou et al., 1997). The invasive phenotype displayed by CTB is associated with the changes in the regulation of ECM ligands and their integrin receptors. CTB villous stem cells in contact with the basement membrane at sites of column formation express $\alpha_\beta_3$ laminin receptors and also express $\alpha_\beta_6$ and $\alpha_\beta_6$ integrins. As CTB differentiate, $\alpha_\beta_3$, $\alpha_\beta_6$ and $\alpha_\beta_6$ integrins are no longer expressed and there is a sequential up-regulation of fibronectin $\alpha_\beta_6$ receptor in cell columns (Damsky et al., 1992) and $\alpha_\beta_6$ laminin/collagen receptor in the uterine wall (Vicovac et al., 1995). Although nothing is known about the signals that promote this differentiation program, function-perturbing antibodies in conjunction with in vitro models have demonstrated that interactions of $\alpha_\beta_3$ integrin with fibronectin restrain invasion, and that $\alpha_\beta_6$ interaction with collagen IV and laminin can promote CTB invasion (Damsky et al., 1994). In parallel, $\alpha_\beta_3$ integrin expression that was not detected in undifferentiated villous CTB has been reported to become up-regulated in invading CTB within the uterine wall and vasculature. Perturbing the function of $\alpha_\beta_3$ integrin affects CTB invasion in vitro (Zhou et al., 1997) indicating that this receptor, like the $\alpha_\beta_3$ integrin, contributes significantly to the invasive phenotype of CTB. Hence, a switch in integrin expression maintains a balance in the invasion-restricting and invasion-promoting mechanisms of CTB into the uterine wall of the mother and may aid to develop and maintain the architecture of the placenta and possibly activate specific signal transduction pathways that promote the survival of the developing fetus.

Fetal membranes undergo structural and morphological changes during parturition, yet the exact mechanism of fetal membrane rupture during labour still remains a matter of controversy and speculation (Novak, 1991). Morphological examination of the amnion suggests that the membrane undergoes localized weakening and degeneration with reduced cytotrophoblast and decidual thickness at a specific point overlying the cervix (McLaren et al., 2000). Digestion of ECM of the fetal membranes occurs during parturition and is carried out largely by locally produced MMP (Xu et al., 2002). Changes in the activity of proteinases, e.g. plasminogen activator (uPA) and MMP in the amniotic fluid and gestational tissues are associated with the onset of labour and rupture of fetal membranes (Athayde et al., 1998). Cytokines are involved in inducing increased levels of MMP expression and activity in the membranes-associated with rupture (Bowen et al., 2002). Induction of IL-1, IL-6, TNF-$\alpha$, pro عبدالله غلاطين E2 and F2 in the fetal membranes during parturition is well documented (Bowen et al., 2002). TNF-$\alpha$ stimulates production of MMP and uPA by chorio (So et al., 1992) while treatment of amniochorion explants with lipopolysaccharide, a stimulator of cytokine-release, also results in increased synthesis and release of MMP-2 (Fortunato et al., 1996). The pattern of production of certain cytokines is altered in the fetal membranes during normal term parturition (Bowen et al., 2002). Even though the cytokines influence changes in the proteolytic activity within the membranes (Bowen et al., 2002) resulting in ECM degradation, it is still not known how these changes can result in rearrangement of ECM and integrin expression.

In this study, we demonstrate significant expression of epithelial-restricted, tumour-associated $\alpha_\beta_3$ integrin in the epithelial layer of amniotic membranes of women at term. We also demonstrate weak staining of the integrin in the chorionic epithelium and at the decidua edge. The expression of $\alpha_\beta_3$ integrin in term fetal membranes of NVD correlated with the expression of both pro-MMP-9 and pro-MMP-2 whereas only pro-MMP-2 expression was observed in the fetal membranes of term CS. These data suggest that $\alpha_\beta_3$ integrin expression occurs in the epithelium of fetal membranes at term and that the induction of the pro-MMP-9 expression in association with $\alpha_\beta_3$ integrin may be required to initiate the rupture of fetal membranes to induce labour.

Materials and methods

This project was approved by the Human Ethics Committee of the Mercy Hospital for Women, Melbourne, Australia. Human term placenta, chorion and amniotic tissues were collected from women at 38–40 weeks gestation from the following labour groups: (i) from women in spontaneous labour and normal vaginal delivery and (ii) from women undergoing elective CS due to cephalopelvic disproportion, breech presentation and/or previous CS. The mean gestation period of pregnancy for term normal deliveries was 40 ± 1.48 weeks and for term CS but not in labour was 39.2 ± 0.39 weeks. The birthweight for term normal deliveries was 3231 ± 476 g while for CS deliveries was 3400 ± 275 g. The women included in the study delivered healthy, singleton infants and were free from infection during the term of the pregnancy. Tissues were received in the laboratory within 20 min of delivery and the surface amnion–chorion membranes were carefully separated from the underlying tissue. Placental tissues were dissected from the basal plate and rolled on a plastic rod. The tissues were frozen in cylinders of OCT compound (Tissue-Tek, Japan) using iso-pentane cooled in dry ice. The tissues were stored at −80°C until needed.

Frozen sections were cut at 5 μm and mounted onto poly-t-lysine coated slides. The cut sections were stored at −20°C until required. For the demonstration of antigen expression, sections were fixed in cold acetone for 15 min and held in Tris–saline buffer (TBS) pH 7.6. Endogenous peroxidase activity was removed using 3% H2O2 in methanol for 10 min. The sections were incubated for 1 h in $\alpha_\beta_3$ monoclonal antibody (E7P; Chemicon, USA) diluted 1:200 in 1% bovine serum albumin (BSA) in TBS (100 mmol/l, pH 7.6). Antibody binding was amplified using Dako LSAB+ biotin and streptavidin horseradish peroxidase (Dako, USA) for 15 min each and the complex was visualized using diaminobenzidine. Nuclei were counterstained using Mayer’s haematoxylin and the sections mounted and coverslipped using a resinous mounting agent. An isotype IgG1 suitably diluted was substituted for the antibody as a negative control and sections of ovarian tumour known to express $\alpha_\alpha_6$ integrin were used as a positive control (Ahmed et al., 2002c).

Staining assessment

Sections were assessed microscopically for positive DAB staining. The staining was scored blind for both extent and intensity of staining. The entire tissue section was scored and the extent of staining was determined on a scale of 0–5 according to the estimated percentage of cells stained: 0 < 10%; 1 = 11–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–90%; 5 > 90% (Armes et al., 1999).

Staining intensity was assessed on a scale of 0–3: 0 = no staining; negative; 1 = pale brown; weak; 2 = brown; moderate; 3 = dark brown, strong (Armes et al., 1999).

Preparation of fetal membrane homogenate

Frozen fetal membranes (amnion or chorion) were cut into small pieces (0.1 mg) and homogenized in Tris–HCl buffer [10 mmol/l Tris, 150 mmol/l NaCl, 2 mmol/l EGTA, 2mmol/l dithiothreitol, 1 mmol/l orthovanadate, 1 mmol/l phenylmethylsulphonyl fluoride, 5 μg/ml aprotinin, pH 7.0] by repeated uniform strokes (~6). Samples were centrifuged at 10 000 g for 20 min. The supernatant was recovered and relative protein concentration was determined. At this stage the samples can be stored at −80°C and are stable for months.
Protein assay

Total protein content was determined using a commercial protein assay kit with BSA standards according to the manufacturer’s instruction (Pierce, USA).

Western blot analysis

Fetal membrane homogenates containing equal amounts of protein were electrophoresed on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gels under non-reducing conditions and transferred to nitrocellulose membranes. Membranes were probed with immunoaffinity-purified monoclonal anti-αvβ6 (Calbiochem, USA) followed by peroxidase-labelled secondary antibody and visualized by the ECL (Amersham, UK) detection system according to the manufacturer’s instructions. Cell lysate from β6 integrin transfected oral squamous carcinoma cell line VB6 was used as β6 integrin control (Thomas et al., 2001a).

Zymography

Pro-MMP-2 and pro-MMP-9 expression in fetal membrane homogenates was analysed using 10% SDS–gelatin (1 mg/ml final concentration) zymography under non-reducing conditions as described previously (Ahmed et al., 2002c). Gelatinolytic activity attributed by MMP-2 and MMP-9 was confirmed by activation with APMA (2 mmol/l) for 4 h prior to zymography. Activation of pro-MMP-2 and pro-MMP-9 could be abolished by incubating zymograms with 1:10 phenanthroline (2 mmol/l) or EDTA (data not shown).

Statistical analysis

For immunohistochemistry, the association between the extent and intensity of immunostaining with the tissue type, i.e. normal vaginal delivery versus elective CS, was determined by χ² analyses using SPSS statistical package (Coakes and Steed, 1995). Student’s t-test was used for other statistical...

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**Table I.** Extent and intensity of staining of αvβ6 integrin in the amnion and chorion obtained after normal vaginal delivery (NVD) and caesarean section (CS)

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Scoring: 10 fetal membranes were scored in each group.

Extent of staining was scored as described in Materials and methods. There was no significant difference in the extent of staining between NVD and CS (χ² = 0.2667, P = 0.875).

Intensity of staining was determined as described in Materials and methods. There was a significant difference in the intensity of staining between NVD and CS (χ² = 10.2545, P = 0.0059).

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Figure 1. Immunohistochemical expression of αvβ6 integrin in the amnion and chorion obtained from women after normal vaginal delivery (NVD) and caesarean section (CS). Cryosections of the membranes were stained by the immunoperoxidase method for the expression of αvβ6 integrin as described in Materials and methods. (a) Haematoxylin and eosin staining of amnion and chorion obtained after NVD; (b) αvβ6 expression in the amnion and chorion after NVD; (c) αvβ6 expression in the amnion and chorion after CS; and (d) αvβ6 expression in the chorion after CS.
Figure 2. Expression of α₆β₃ integrin in (a) amnion and (b) chorion obtained from women after normal vaginal delivery (NVD) and caesarean section (CS). Fetal membrane homogenates were prepared and western blotting was performed as described in Materials and methods. A total of 17 µg of protein was loaded in a total volume of 25 µl in each lane. The results are representative of one experiment repeated three times. (c) Quantification of α₆β₃ integrin expression was performed by densitometry and expressed as mean peak optical density ± SEM of the number of samples described in each group. Results are representative of one experiment. The experiment was repeated three times.
analyses. Statistical significance was indicated by $P < 0.05$. Data are presented as means ± SEM.

Results

Immunohistochemical localization and analysis of $\alpha\beta_6$ integrin expression in term fetal membranes

Sections were assessed microscopically for positive DAB staining. The staining of $\alpha\beta_6$ integrin expression was scored in a blind fashion. Staining was scored by (i) the extent of staining on a scale of 0–5 according to the estimated percentage of positively stained cells; and (ii) the intensity of staining on a scale of 0–3. Four sections were assessed per tissue and data were pooled and averaged for each tissue. Parallel frozen sections were stained with haematoxylin and eosin to confirm the morphology of the tissue. Immunostained sections were reviewed independently by two pathologists to verify immunohistochemical score.

As presented in Table I, all membranes obtained either after NVD or CS ($n = 20$) exhibited staining for $\alpha\beta_6$ integrin. The staining was confined to the epithelial layer of the amnion and was not detected in the mesenchymal layer. Although there was no difference in the extent of staining, significantly enhanced staining intensity was present in NVD amniotic epithelium compared to epithelium associated with the amnion of CS patients ($\chi^2 = 10.2545$, $P = 0.0059$) (Figure 1 and Table I). All chorion specimens exhibited weak $\alpha\beta_6$ integrin staining (1 = pale brown) (Figure 1 and Table I). The staining was demonstrated in the chorionic epithelium as well as at the decidua edge, and there was no significant difference between the NVD and CS groups. No positive staining was observed in the basement membrane nor the mesothelial layer of the membranes. The trophoblasts obtained from NVD and CS women showed no staining for $\alpha\beta_6$ integrin.

To further evaluate the expression of $\alpha\beta_6$ integrin in placental membranes, fetal membrane homogenates were prepared and western blot analysis was performed using equal protein load (Figure 2). The expression of $\alpha\beta_6$ integrin in the chorion of NVD and CS women was significantly lower than in the amnion ($P < 0.01$). No difference in the expression of integrin was observed in the chorion between the NVD and CS groups and was consistent with the immunohistochemical analysis. Western blots of the amnion homogenates from NVD and CS

Figure 3. Gelatin zymography showing the amounts of pro-matrix metalloproteinase-2 (pro-MMP-2) and pro-MMP-9 in (a) amnion and (b) chorion membrane homogenates. The positions of purified pro-MMP-2 and pro-MMP-9 are shown on the left. A total of 17 μg of protein was loaded in a total volume of 25 μl in each lane. Results are representative of three independent experiments. NVD = normal vaginal delivery; CS = caesarean section.
women showed no difference in the total expression of $\alpha_\beta_6$ integrin. These results are consistent with that of immunohistochemistry that showed no difference in the extent (which represents total number of cells) of $\alpha_\beta_6$ staining in the amnion of NVD and CS women. However, the significant increase in the intensity of staining for $\alpha_\beta_6$ integrin in the amniotic epithelium of NVD women compared to the CS group by the immunohistochemical method may be due to differences in the epitope recognition sites of the antibody used in the method. One would assume that the results from the immunohistochemical method would demonstrate immunostaining of intact antigens exposed on the surface of the membranes, whereas western blot analysis would show the presence of total antigen in the membrane homogenates. These results suggest that, even though the total $\alpha_\beta_6$ integrin content of the amnion at term remains unchanged between the NVD and CS groups, there is a significant enhancement in the surface expression of $\alpha_\beta_6$ integrin in amnion in labour.

**Expression of pro-MMP-2 and pro-MMP-9 in term fetal membranes**

In order to correlate the potential role of $\alpha_\beta_6$ integrin in the regulation of proteolytic activity associated with labour, we examined the expression of pro-MMP-2 and pro-MMP-9 in the fetal membrane homogenates obtained from term NVD and CS women (Figure 3). Gelatin zymography showed expression of pro-MMP-2 and pro-MMP-9 in the amnion and chorion obtained from NVD patients, whereas the expression of pro-MMP-2 was only observed in fetal membrane homogenates obtained from the CS group. Collectively, these data demonstrate that term amnion and chorion express pro-MMP-2 but that the expression of pro-MMP-9 is only induced in membranes with the induction of labour.

**Discussion**

The invasive phenotype of human placenta is manifested predominantly during the first half of gestation, when a subset of chorionic CTB leave the fetal compartment and invade the uterine wall and maternal spiral arteries. Development of this invasive phenotype is accompanied by a comprehensive transformation in the expression of CTB adhesion molecules (Zhou et al., 1997). It is at this stage of uterine invasion that the expression of $\alpha_\beta_6$ integrin is observed in the CTB villous cells documenting a specific association of this integrin with the invasive process. However, the expression of this integrin is lost during the second trimester of gestation (Zhou et al., 1997). There is a paucity of data concerning the expression of $\alpha_\beta_6$ integrin in placenta and fetal membranes in the later stages of pregnancy.

In this study, we report the expression of $\alpha_\beta_6$ integrin in term fetal membranes and demonstrate enhanced surface expression of the integrin in amniotic epithelium of the membranes obtained from women after NVD. We also show that fetal membranes from CS patients only express pro-MMP-2 whereas both pro-MMP-9 and pro-MMP-2 are expressed in women undergoing NVD. These results suggest involvement of an MMP-9-mediated proteolytic system in the fetal membranes of NVD women that may be requisite for membrane rupture and initiation of labour.

Basal expression of $\alpha_\beta_6$ integrin was present in all chorionic membranes and it was present both in the epithelium and at the decidual edge. There was no significant difference in the expression of $\alpha_\beta_6$ integrin in the chorion obtained from women undergoing NVD to that obtained from women undergoing CS. No expression of $\alpha_\beta_6$ integrin was observed in placental trophoblasts. On the other hand, expression of $\alpha_\beta_6$ integrin was significantly higher in all amnion than in chorion. Even though there was no significant difference in the total expression of $\alpha_\beta_6$ integrin in the amniotic epithelium between the NVD and CS groups, there was significant enhancement in the intensity of integrin expression in the NVD group compared to those who underwent CS.

To date, most of the literature describing integrins during gestation has focused on their involvement in the early stages of pregnancy, i.e. implantation and placentation (Bowen and Hunt, 2000). Changes in the expression and/or function of the integrin receptors have been reported in the first and second trimester of pregnancy (Zhou et al., 1997). It has been suggested that synthesis or loss of expression/function of these and other adhesion molecules is favoured by a selection process modulated by the ECM composition and contributes to the tissue remodelling process associated with the normal development of the placenta (Bowen and Hunt, 2000). Under normal physiological conditions, very low basal expression of the integrin $\alpha_\beta_6$ is present and is restricted to epithelial cells (Breuss et al., 1993). The expression of the integrin is dramatically induced in response to tissue injury (Haapasalmi et al., 1996) where the integrin binds to and activates latent extracellular complexes of the anti-inflammatory, pro-brotic cytokine, transforming growth factor-$\beta_1$ (Munger et al., 1999); (Pittet et al., 2001). Under normal physiological conditions, $\alpha_\beta_6$ integrin has a unique role of regulating the action of extracellular inactive TGF-$\beta_1$ at intracellular sites where it is activated by the integrin and thereby limits the undesirable consequences of free diffusion (Pittet et al., 2001). In carcinomas, the expression of $\alpha_\beta_6$ integrin has been associated with growth promotion (Agrez et al., 1994) and ECM degradation through proteolytic activation of MMP-9 (Ahmed et al., 2002b). We and others have recently shown inhibition of growth (Xue et al., 2001) and MMP-9 secretion and ECM degradation by blocking $\alpha_\beta_6$ integrin function in ovarian, colon and oral squamous carcinomas (Agrez et al., 1999, Thomas et al., 2001b; Ahmed et al., 2002a). These data suggest that $\alpha_\beta_6$ integrin not only regulates growth but also modulates proteolytic events through MMP-9 activation. This invasive aspect of $\alpha_\beta_6$ integrin is of great significance in human parturition. Successful delivery is associated with extensive ECM remodelling of the uterus, cervix and fetal membranes (Bowen et al., 2002). During this process the tensile strength of the fetal membranes is weakened and there is an intensive disruption of the constituents in ECM associated with the rupture of the fetal membranes (McLaren et al., 2000). MMP-2 and MMP-9 can degrade collagen IV, a major constituent of the ECM and basement membrane. These two MMP have been identified in fetal membranes and an increase in MMP-9 content in the fetal membranes has been associated with term labour (Xu et al., 2002). A recent study has shown induction of MMP-9 expression in amnion adjacent to the cervix at the onset of labour and has supported its role in the rupture of amniotic membranes (McLaren et al., 2000). As the proteolytic events in tissue remodelling are similar in tumorigenesis, wound healing and parturition, it is not surprising that the expression of $\alpha_\beta_6$ integrin correlates with MMP-9 expression in fetal membranes with the onset of labour. As the expression of the integrin has been shown to regulate invasion and migration of tumour cells (Xue et al., 2001), and keratinocytes in wound healing (Haapasalmi et al., 1996), it is not unreasonable to postulate that the integrin may have the same function in the amnion during labour. It is also possible that $\alpha_\beta_6$ integrin expressed in the amnion–chorionic cells have a functional role in growth promotion and diffusion of water-soluble substances. Considering the data presented in this study and the known role of $\alpha_\beta_6$ integrin in tumour progression and wound healing, it is possible that the integrin may have a fundamental role in labour-associated increase in MMP-9-mediated proteolytic activity required for fetal membrane rupture. The present study raises the possibility that the expression of $\alpha_\beta_6$ integrin in association with pro-MMP-9 may be a
marker of impending labour and may have a clinical significance in relation to the incidence of premature membrane rupture.

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