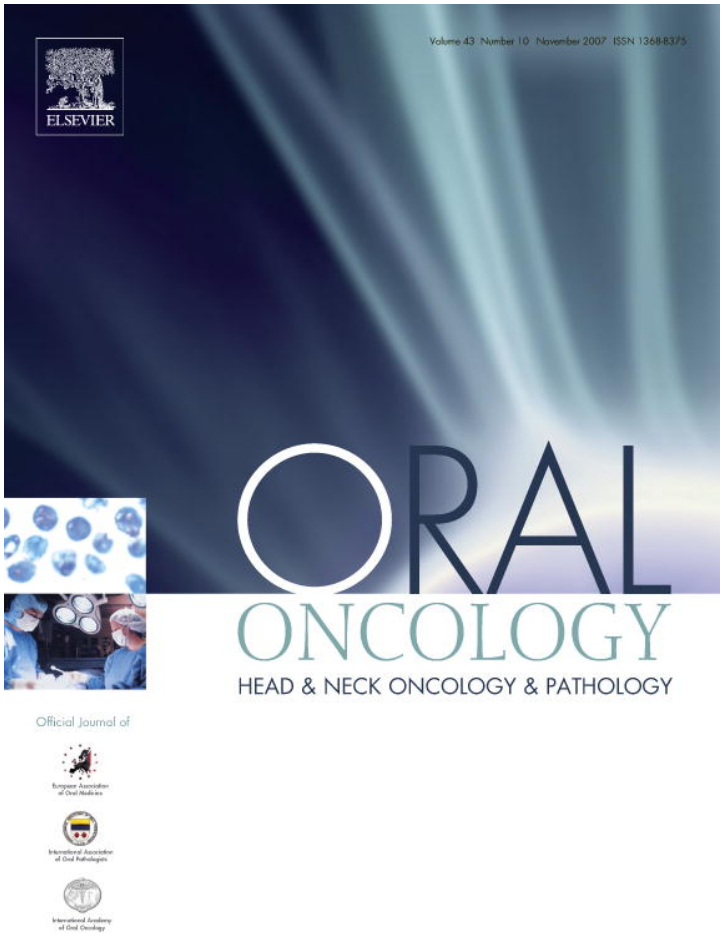

Oral Oncology (2007) 43, 987–998

available at www.sciencedirect.com

ScienceDirect

journal homepage: http://intl.elsevierhealth.com/journals/oron/

ORAL ONCOLOGY

Malignancy-related 67 kDa laminin receptor in adenoid cystic carcinoma. Effect on migration and β-catenin expression

Vanessa Morais Freitas [a], Letícia Nogueira da Gama de Souza [a], Elaine Cyreno Oliveira [a], Cristiane Furuse [b], Vera Cavalcanti de Araújo [b], Ruy Gastaldoni Jaeger [a,*]

[a] *Department of Cellular and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil*
[b] *Department of Oral Pathology, São Leopoldo Mandic Dental Research Institute, Campinas, SP, Brazil*

Received 1 September 2006; accepted 8 November 2006
Available online 25 January 2007

KEYWORDS
Adenoid cystic carcinoma;
Laminin;
67LR;
β-Catenin;
Cell migration

Summary Adenoid cystic carcinoma is a malignant salivary gland neoplasm with recurrence and metastasis. We studied the expression of a malignancy-related non-integrin laminin receptor, the 67LR, in this neoplasm. Immunohistochemistry showed 67LR in adenoid cystic carcinoma. This receptor binds a sequence of laminin β1 chain, the YIGSR peptide. We studied the effect of 67LR and YIGSR in cells (CAC2) from adenoid cystic carcinoma. Three-dimensional cultures of cells embedded into either laminin-111 gel (controls) or YIGSR-enriched laminin-111 (treated) were prepared and studied by light microscopy. CAC2 cells treated with YIGSR appeared fibroblast-like, while control cells were epithelioid. Blockage of 67LR by antibody abolished YIGSR effect in three-dimensional cultures. We analysed the relevance of 67LR and YIGSR on β-catenin expression in CAC2 cells. Immunofluorescence and immunoblot showed that YIGSR decreased β-catenin, while blockage of 67LR restored the presence of this molecule. The 67LR and YIGSR induced fibroblast-like morphology in CAC2 cells, with disruption of cell–cell contacts and decrease of β-catenin. These features resemble epithelial–mesenchymal transition (EMT). EMT also increases cell migration. In monolayer assays YIGSR increased migration of CAC2 cells. We conclude that 67LR and YIGSR are involved in epithelial–mesenchymal transition, modulation of β-catenin expression, and migratory activity of CAC2 cells.

* Corresponding author. Tel.: +55 11 30918034; fax: +55 11 30917402.
E-mail address: rgjaeger@usp.br (R. Gastaldoni Jaeger).

1368-8375/$ - see front matter
doi:10.1016/j.oraloncology.2006.11.005

Introduction

Adenoid cystic carcinoma is a frequent malignant salivary gland neoplasm with recurrence and metastasis.[1] A prominent feature of this tumour is its affinity for basement membrane-rich tissues, such as nerves and blood vessels.[1,2]

This neoplasm is histologically characterised by a sheet or island-like proliferation of round or cuboidal epithelial cells, with scanty cytoplasm and hyperchromatic large oval nucleus.[1] Growth patterns are solid, tubular and pseudocystic. Perineural invasion is a common histological finding of adenoid cystic carcinoma.[1,2] Electron microscopy and immunohistochemistry demonstrated a conspicuous basement membrane in this neoplasm.[3–6]

It has been suggested that basement membrane proteins play an important role as regulatory factor of phenotypic differences among salivary gland neoplasms.[7–14] We have demonstrated that laminin modulates the morphology and protease activity of an adenoid cystic carcinoma cell line (CAC2 cells).[11,12] We have also shown that integrins play a part regulating this effect.[11,15] We are currently studying the role played by non-integrin receptors on adenoid cystic carcinoma.

Among the non-integrin receptors the 67LR is a 37/67 kDa laminin-binding protein that selectively binds a sequence of the laminin β1 chain, the YIGSR peptide.[16,17] This receptor is overexpressed on the surface of a variety of tumour cells.[18–22] The strong correlation between increased 67LR expression and the metastatic potential of tumour cells suggest that the receptor plays a role in development of the metastatic phenotype.[20,21,23,24]

We are studying the expression of the 67LR in the adenoid cystic carcinoma in vivo and in vitro. We created a model system to study the effect of this receptor and its ligand, the peptide YIGSR, in a cell line (CAC2) derived from human adenoid cystic carcinoma. Our results showed that 67LR is expressed in adenoid cystic carcinoma. Furthermore, our in vitro results suggest that the complex 67LR–YIGSR is involved in epithelial–mesenchymal transition, modulation of β-catenin expression and increase of migratory activity of CAC2 cells.

Materials and methods

Immunolocalisation of laminin-111 and the 67LR receptor in adenoid cystic carcinoma in vivo

The 67LR binds the peptide YIGSR, located at the β chain of the laminin-111 isoform (former laminin-1, new classification proposed by Aumailley et al.[25]). For this reason we searched for laminin-111 and the 67LR in our samples.

Ten cases of adenoid cystic carcinoma were retrieved from the files of the Department of Oral Pathology, São Leopoldo Mandic Dental Research Institute, Campinas, SP, Brazil. Growth patterns were cribriform (six cases), tubular (two cases), and solid (two cases). Formalin-fixed paraffin-embedded tissues were studied by immunohistochemistry. Sections (3 μm) were obtained and subjected to the EnVision method (EnVision, Dako Corp., Carpinteria, CA, USA). Sections mounted on 3-aminopropyltriethoxy-silane coated slides (Sigma Chemical Corp., St. Louis MO, USA) were dewaxed in xylene and hydrated in graded ethanol. Endogenous peroxidase activity was inhibited by incubation of the sections in 3% H_2O_2 in PBS for 20 min. Antigen retrieval was carried out by treating sections with 1% pepsin in 10 mM HCl for 1 h at 37 °C. Sections were then blocked with 1% bovine serum albumin (BSA, Sigma) in Tris–HCl. A rabbit antiserum to laminin-111 (kindly provided by Dr. Hynda Kleinman, NIDCR, NIH, USA) was used diluted 1:50 in Tris–HCl. Diaminobenzidine (Sigma) was used as the chromogen and the sections were counterstained with Mayer's haematoxylin (Sigma). Replacement of primary antibodies by rabbit non-immune serum served as negative controls.

A similar protocol with no antigen retrieval was carried out to investigate the presence of the 67LR receptor in adenoid cystic carcinoma. A rabbit polyclonal antibody was kindly provided by Dr. Hynda Kleinman (NIDCR, NIH, USA). This antibody was made against a bacterial fusion protein coded for by the β-galactosidase gene plus the 0.9-kb cDNA sequence encoding the nearly entire molecule of the 67LR and recognises the 37 kDa precursor and the 67-kDa laminin receptor.[26,27] Antibody was diluted 1:100 in Tris–HCl.

Cell culture

We used a cell line (CAC2) derived from a human adenoid cystic carcinoma. The characterisation of CAC2 cells was published elsewhere.[11,12] These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented by 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antibiotic-antimycotic solution (Sigma). The cells were maintained in 25 cm^2 flasks in a humidified atmosphere of 5% CO_2 at 37 °C.

Immunofluorescence

Immunofluorescence was performed to detect laminin-111 and the 67LR in CAC2 cells. For laminin-111 analysis cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, rinsed in PBS, incubated with 1% BSA in PBS, and stained with rabbit antiserum against laminin-111 (1:50 in PBS). Primary antibody was revealed by an anti-rabbit Cy3 conjugated secondary antibody (Zymed-Invitrogen Co., Carlsbad, CA, USA). All incubations were carried out for 60 min at room temperature and Pro Long (Invitrogen-Molecular Probes, Eugene, OR, USA) was used as a mounting medium. Nuclei were counterstained with either DAPI or Sytox Green (Invitrogen-Molecular Probes). Replacement of the primary antibody by PBS was used as negative control. The same protocol was carried out to investigate the presence of the 67LR receptor in CAC2 cells. The antibody was the same described before and was diluted 1:500 in PBS. Primary antibody was revealed by an anti-rabbit Cy3 conjugated secondary antibody (Zymed-Invitrogen). Nuclei were counterstained with either DAPI or Sytox Green (Invitrogen-Molecular Probes). Replacement of the primary antibody by PBS was used as negative control.

Three-dimensional culture of CAC2 cells embedded into either laminin-111 gel or YIGSR-enriched laminin-111

This assay was carried out to study the effect of laminin-111 and its peptide YIGSR in CAC2 cells. We also analysed the role of the 67LR receptor in the morphology of CAC2 cells.

We used a laminin-111 gel in DMEM (1 mg/ml, Trevigen Inc., Gaithesburg, MD, USA). This preparation will be referred throughout the text as ''laminin-111 gel''. Three-dimensional (3D) cultures were prepared by growing CAC2 cells to confluence as monolayers, followed by trypsinisation and embedment into laminin-111 gel as single cells (2×10^5 cells/ml). The laminin-111 gel containing CAC2 cells was then dispensed into cryovials and maintained at 37 °C with 5% CO_2. Cells were grown within this three-dimensional matrix of laminin-111 for 48 h. Three-dimensional cultures followed the protocol described by Weaver et al.[28], with some modifications.[11,12]

We also prepared a mixture of laminin-111 gel and YIGSR (Sigma Chemical Co., St. Louis, MO, USA). The peptide was diluted in ultrapure water (1 mg/ml). After that, YIGSR was mixed with laminin-111 gel, to reach a final concentration of 50 μg/ml. This preparation will be referred throughout the text as ''YIGSR-enriched laminin-111''. Three-dimensional (3D) cultures were prepared by growing CAC2 cells to confluence as monolayers, followed by trypsinisation and embedment into YIGSR-enriched laminin-111 as single cells (2×10^5 cells/ml). The YIGSR-enriched laminin-111 containing CAC2 cells was then dispensed into cryovials and maintained at 37 °C with 5% CO_2. Cells were grown within this three-dimensional matrix for 48 h. CAC2 cells grown inside laminin-111 gel served as controls.

Cells grown within YIGSR-enriched laminin-111 would interact with an increased amount of YIGSR compared to cells embedded into laminin-111 gel. Thus, this amount of YIGSR is likely to induce different phenotypic changes in CAC2 cells compared to the effect of laminin-111 itself.

We carried out experiments blocking the 67LR with the rabbit polyclonal antibody, followed by growth of CAC2 cells embedded into three-dimensional matrices of YIGSR-enriched laminin-111. The rationale of this assay was to observe whether blockage of 67LR would prevent the effect of YIGSR in CAC2 cells. CAC2 were harvested from the flasks by 0.2% EDTA in PBS (Versene solution) and incubated with antibody against 67LR (1/20 in PBS) for 1 h at 37 °C. CAC2 cells incubated with anti-human IgG (Chemicon-Millipore Co., Billerica, MA, USA) served as controls. Cells with receptor blocked by the 67LR antibody were embedded into YIGSR-enriched laminin-111 as single cells (2×10^5 cells/ml), dispensed into cryovials and maintained at 37 °C with 5% CO_2. CAC2 cells were grown within this three-dimensional matrix for 48 h.

Three-dimensional experiments are summarised in Figure 1A. Treated and control samples were studied by light microscopy.

Light microscopy of CAC2 cells grown in three-dimensional matrices

Treated and control samples were fixed in 10% formalin for 24 h. Even after fixation, the laminin-111 gel used in our preparation was too soft to be embedded directly in paraffin. To circumvent this problem, we dehydrated and embedded the samples in Histogel (Perk Scientific Inc., Devon, PA, USA).[11] Heating the Histogel to 50 °C converts the gel into a liquid state, which allows infiltration of the samples. After the infiltration, the Histogel converts back into a solid as it cools. Final hardening is achieved at room temperature. Since Histogel is in aqueous media, the samples were then dehydrated again, paraffin-embedded and stained by haematoxylin–eosin (H&E).

Three-dimensional sample preparation is summarised in Figure 1B.

Effect of YIGSR and the 67LR on β-catenin expression in CAC2 cells

We analysed whether YIGSR would inhibit the expression of β-catenin in CAC2 cells. Three-dimensional cultures of CAC2 cells embedded into either laminin-111 gel or YIGSR-enriched laminin-111 were prepared as described before. Furthermore, CAC2 cells with the 67LR receptor blocked by the specific antibody were grown inside YIGSR-enriched laminin-111. All these samples were subjected to immunoblot. Cells were lysed in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) with protease inhibitor cocktail (Sigma). Lysates were centrifuged (10,000*g*) for 10 min at 4 °C. The supernatants were recovered, quantified (BCA kit, Pierce Biotechnology Inc., Rockford IL, USA) and resuspended in Laemmli sample buffer containing 62,5 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% mercaptoethanol, 0,001% bromophenol blue. Equal amounts (20 μg) of cell lysates were applied to gels based on protein content. CAC2 cells lysates were electrophoresed in 4–12% polyacrylamide gradient gels. Proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham), blocked in TBS with 2.5% nonfat dry milk overnight at 4 °C. Following one wash in TBS with 0.05% Tween 20 (TBST), the membrane was probed with the antibody against β-catenin (rabbit polyclonal from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by HRP-conjugated secondary antibodies (1:10,000), and developed using an ECL chemiluminescent substrate (Amersham).

We also performed whole mount immunofluorescence to detect β-catenin in CAC2 cells embedded into either laminin-111 gel or YIGSR-enriched laminin-111. Cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, rinsed in PBS, and permeabilised with 0.5% Triton X-100 in PBS. Samples were incubated with 1% BSA in PBS, and stained with rabbit antibody against β-catenin (rabbit polyclonal from Santa Cruz) diluted 1:50 in PBS. Primary antibody was revealed by an anti-rabbit Cy3 conjugated secondary antibody (Zymed-Invitrogen). All incubations were carried out for 60 min at room temperature and Pro Long (Invitrogen-Molecular Probes, Eugene, OR, USA) was used as a mounting medium. Replacement of the primary antibody by PBS was used as negative control. The same protocol was carried out to investigate the presence of β-catenin in CAC2 cells with the 67LR receptor blocked by antibody, followed by growth in YIGSR-enriched laminin-111.

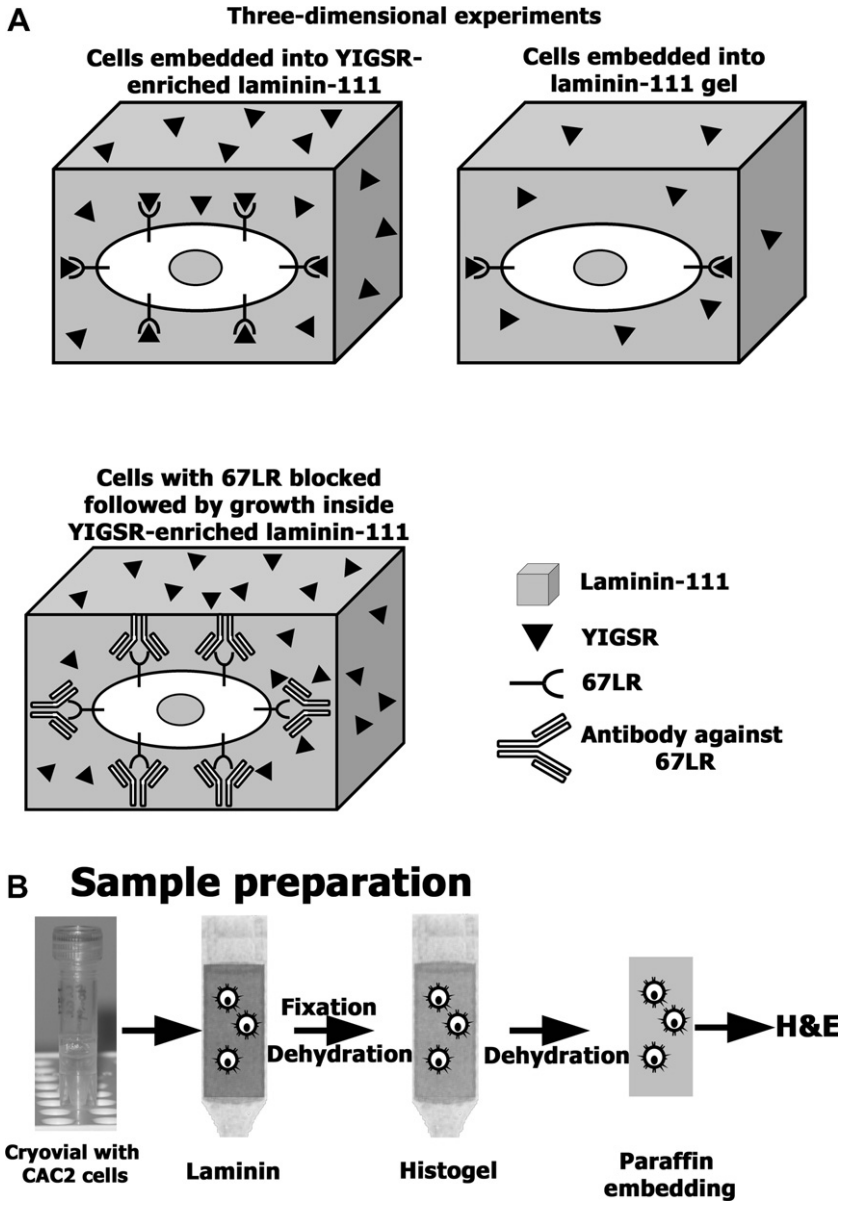

Figure 1 Diagrams summarising the three-dimensional experiments carried out in this study. CAC2 cells embedded into YIGSR-enriched laminin-111 (A, upper left) would interact with an increased amount of YIGSR (black triangles) compared to cells embedded into laminin-111 gel (A, upper right). Thus, this amount of YIGSR is likely to induce different phenotypic changes in CAC2 cells compared to the effect of laminin-111 itself. In the blockage experiment (A, lower left) CAC2 cells were pretreated with the 67LR rabbit polyclonal antibody. Cells with blocked receptors were then embedded into YIGSR-enriched laminin-111. This is a competition assay, designed to observe whether blockage of 67LR would prevent the effect of YIGSR in CAC2 cells. Treated and control samples were dispensed into cryovials and maintained at 37 °C with 5% CO_2 for 48 h (B). Samples were fixed in 10% formalin for 24 h, dehydrated and embedded in Histogel (B). Since Histogel is in aqueous media, the samples were then dehydrated again, paraffin-embedded and stained by haematoxylin—eosin (H&E).

Activation of Wnt pathway in CAC2 cells

β-catenin is an important component of the Wnt signalling pathway.[29] We then decided to study the effect of Wnt pathway in CAC2 cells grown inside laminin-111 gel. These cells were treated by lithium chloride. One of the well characterised cell biologic actions of lithium chloride is the inhibition of glycogen synthase kinase-3 (GSK3) and the consequent activation of canonical Wnt signalling.[30] We prepared three-dimensional cultures of CAC2 cells embedded into laminin-111 gel containing 20 μM lithium chloride. Cells were grown within this three-dimensional matrix for 48 h. CAC2 cells grown within laminin-111 gel served as controls. Treated and control samples were studied by light microscopy as described before.

Migration assay in CAC2 cells treated by YIGSR

Migration of CAC2 cells was investigated through the use of a standard in vitro monolayer wound assay.[31] Cells were

grown in 24-well plates to confluence, and after 24 h of culture in DMEM the confluent monolayer was gently scraped with a pipette tip to create a cell-free area. Thereafter, the medium was washed out and replaced by serum-free DME containing the YIGSR peptide (50 μg/ml). CAC2 cells grown in serum-free DME served as control. A reference point was created on the bottom of the plate in the field of the wound using direct microscopic visualisation. This procedure permitted photographing the identical spot each time. Wound closure was followed after 0 h, 24, and 72 h.

Results

Presence of laminin-111 and the 67LR in adenoid cystic carcinoma in vivo and in vitro

Laminin-111 was present in adenoid cystic carcinoma in vivo (Fig. 2A). Immunohistochemistry detected this protein as a linear structure in the cribriform subtype (Fig. 2A). Laminin-111 also showed a diffuse expression in solid and cribriform areas (not illustrated). The 67LR receptor was found in adenoid cystic carcinoma cells in vivo (Fig. 2C—F). This receptor was expressed in tubular (Fig. 2C), cribriform (Fig. 2D), and solid (Fig. 2E and F) subtypes. Expression of 67LR was more prominent in cribriform and solid growth patterns (Fig. 2D—F). CAC2 cells exhibited both laminin-111 and the 67LR (Fig. 2G and H). Laminin-111 formed a network distributed throughout the cell membrane (Fig. 2G). The 67LR was observed as diffuse dots (Fig. 2H). Immunofluorescence for both laminin-111 (Fig. 2G) and 67LR (Fig. 2H) represented distribution of these proteins on the cell membrane, since samples were not permeabilised. No cytoplasmic staining was observed.

Three-dimensional culture of CAC2 cells embedded into either laminin-111 gel or YIGSR-enriched laminin-111

Light microscopy showed that laminin-111 gel induced CAC2 cells to create solid formations of closely packed epithelioid cells (Fig. 3A). On the other hand, CAC2 cells embedded into YIGSR-enriched laminin-111 showed a completely different pattern. Cells were non-cohesive, spindle shaped and fibroblast-like (Fig. 3B). To address whether the effect of YIGSR was dependent on 67LR, CAC2 cells were pretreated with antibody against this receptor, followed by growth into YIGSR-enriched laminin-111. Blockage of 67LR receptor inhibited YIGSR effect on CAC2 morphology, with restoration of the epithelioid phenotype (Fig. 3C).

67LR and its ligand YIGSR modulate β-catenin expression in CAC2 cells

Laminin-111 gel induced CAC2 cells to create solid formations of epithelioid cells. On the other hand the laminin-derived peptide YIGSR induced a fibroblast-like morphology in CAC2 cells. We then decided to study whether YIGSR would inhibit β-catenin expression in CAC2 cells. We prepared lysates of three-dimensional cultures of CAC2 cells embedded into either laminin-111 gel or YIGSR-enriched laminin-111. Culture conditions were the same described before. Western blot of these lysates demonstrated that YIGSR decreased the amount of β-catenin in CAC2 cells (Fig. 4A, middle lane) compared to cells embedded into laminin-111 gel (Fig. 4A, left lane). Pretreatment of CAC2 cells with anti-67LR restored β-catenin expression (Fig. 4A, right lane). These results were also observed in confocal sections from whole mount preparations labelled to β-catenin (Fig. 4B—D). CAC2 cells embedded into laminin-111 gel exhibited β-catenin located at cell boundaries (Fig. 4B, asterisk). On the other hand, treatment by YIGSR decreased the presence of this adhesion molecule (Fig. 4C). Blockage of 67LR with specific antibody restored β-catenin expression at the cell edges (Fig. 4D, asterisk).

Activation of the Wnt pathway induced CAC2 cells to create a phenotype similar to that observed when cells were treated with YIGSR

The peptide YIGSR induced fibroblast-like morphology and decreased the expression of the adhesion molecule β-catenin in CAC2 cells. β-catenin is a key player of Wnt signalling pathway. Thus we decided to activate Wnt pathway in CAC2 cells grown inside laminin-111 gel. Three-dimensional cultures of cells embedded into laminin-111 containing lithium chloride (LiCl) were prepared and studied by light microscopy. Our results showed that control cells, embedded into laminin-111 gel, were closely packed and epithelioid (Fig. 5A). On the other hand, CAC2 cells treated by lithium chloride were mostly round and non-cohesive (Fig. 5B and C). Spindle-shaped and fibroblast-like cells were also observed (Fig. 5B and C). These results indicate that lithium chloride, an activator of the Wnt signalling pathway, induced CAC2 cells to create a phenotype similar to that observed when cells are embedded into YIGSR-enriched laminin-111.

YIGSR increase migratory activity of CAC2 cells

The peptide YIGSR induced fibroblast-like morphology, and decreased the expression of the adhesion molecule β-catenin. Taken together, these results would indicate that YIGSR might be involved in migration of CAC2 cells. To explore whether YIGSR affects CAC2 cells migration, we performed an in vitro wound closure assay. Cell monolayers were gently scraped with a pipette tip to create a cell-free area. Closure of this wounded cell-free area was followed after 0, 24, and 72 h. The cell-free wound gap of CAC2 control monolayers healed slowly. However, in the presence of YIGSR, the closure of the wounded area was significantly accelerated (Fig. 6). More cells appeared in the wounded gap, which represented enhanced migratory activity towards the wounded area.

The remaining cell-free area at 24—72 h as a percentage of the initial wound area was taken as an index of wound healing (Fig. 6B). In control CAC2 cells, 85% of the wound area remained cell-free at 24 h after wounding. However, in cells treated by YIGSR, cell-free area decreased to 65%. After 72 h of treatment by YIGSR only 35% of the wound area remained cell-free. On the other hand at least 70% of cell-free area was observed in control samples. Similar results were obtained in at least three experiments.

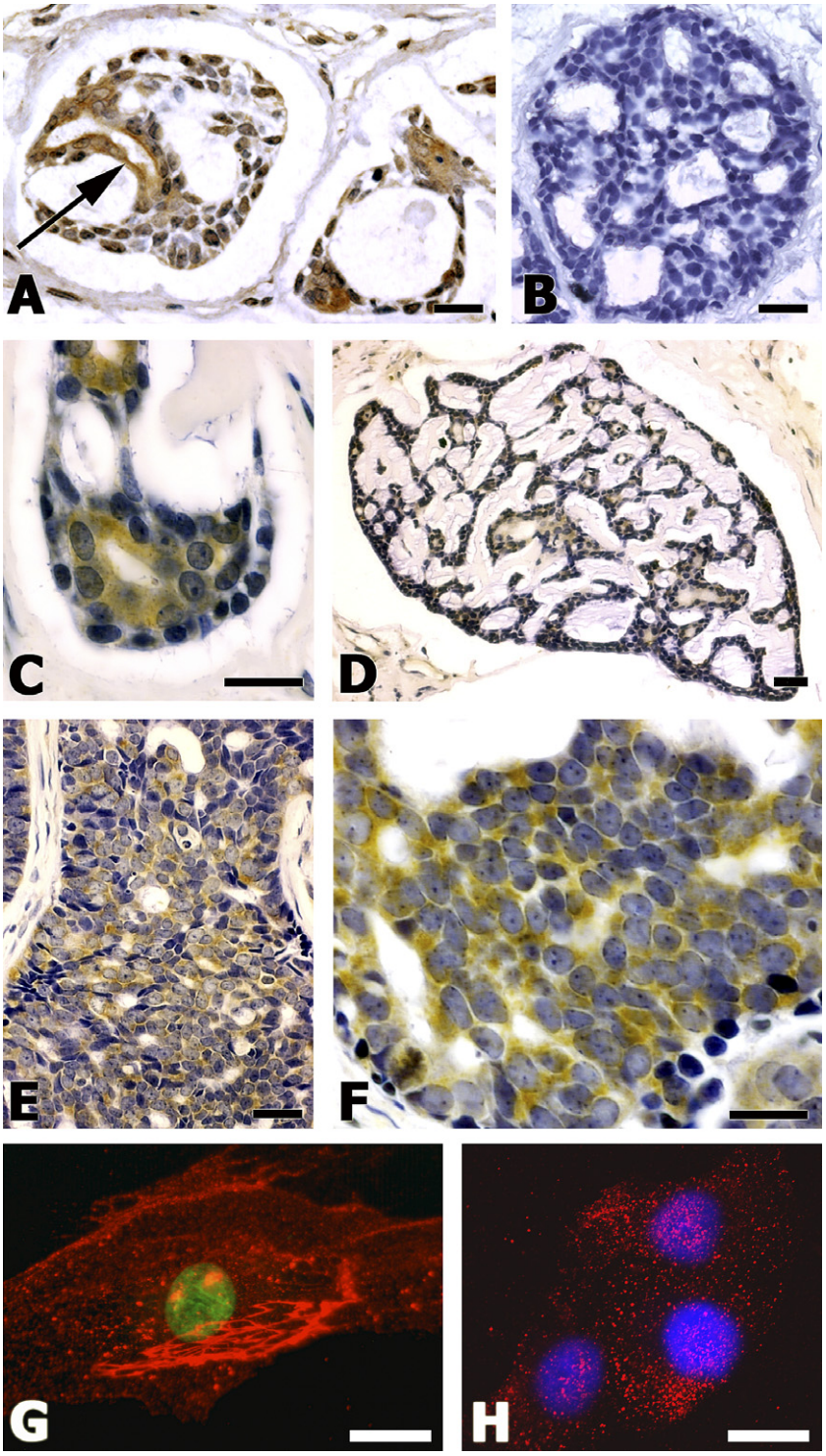

Figure 2 Laminin-111 is detected in adenoid cystic carcinoma in vivo as a linear structure in the cribriform subtype (1A, arrow). Negative controls show no staining in all samples observed (B). The 67 kDa laminin receptor (67LR) is observed in tubular (C), cribriform (D), and solid patterns (E and F). In tubular areas the 67LR is located only at luminal cells (C), while in cribriform and solid patterns the distribution of this receptor is widespread. A cell line (CAC2) derived from adenoid cystic carcinoma expresses laminin-111 as a prominent network and as dots distributed throughout the cell membrane (G). CAC2 cells exhibit the 67LR as diffuse dots (H). Immunofluorescence for both laminin-111 (G) and 67LR (H) represents distribution of these proteins on the cell membrane, since samples were not permeabilised. No cytoplasmic staining is observed. (A–F) Immunoperoxidase technique; (G and H) immunofluorescence of CAC2 cells, where nuclei are counterstained either with Sytox green (G) or with DAPI (H). Scale bar: 20 μm.

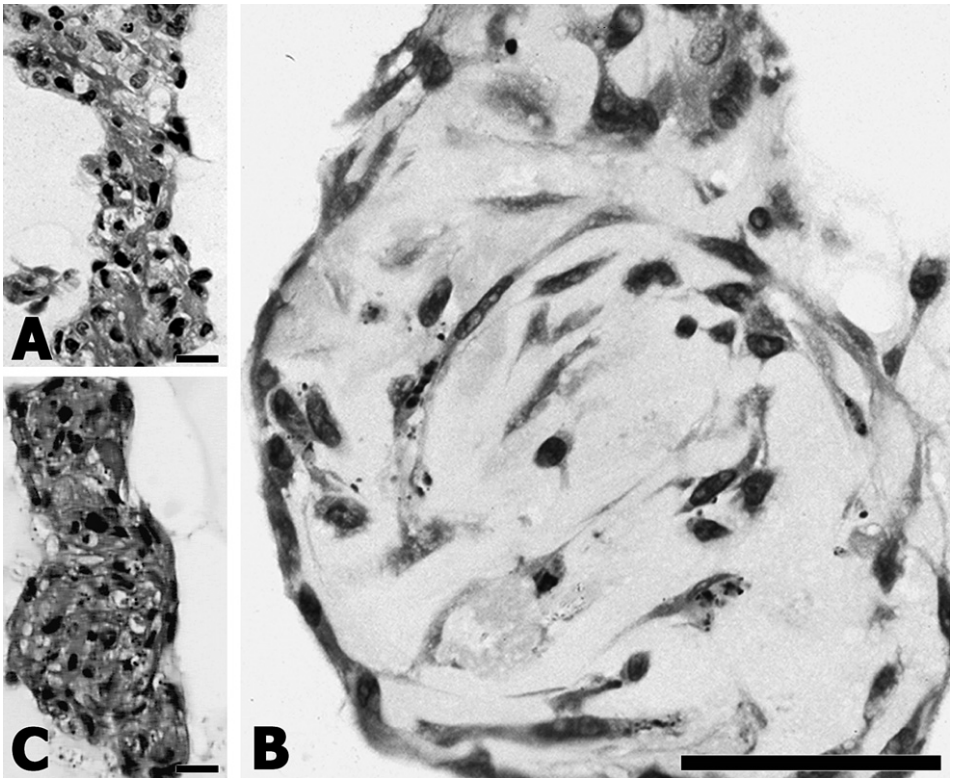

Figure 3 Light microscopy (HE) shows that laminin-111 gel induces CAC2 cells to create solid formations of closely packed epithelioid cells (A). On the other hand, cells grown inside YIGSR-enriched laminin-111 are non-cohesive, spindle-shaped and fibroblast-like (B). Blockage of 67LR receptor inhibited YIGSR effect on CAC2 morphology (C), with restoration of the epithelioid phenotype. Scale bar: 20 μm.

Discussion

Our results show the expression of 67LR in all subtypes of adenoid cystic carcinoma. This expression was more prominent in cribriform and solid subtypes. We also created a model system to study the effect of this receptor and its ligand, the laminin-derived peptide YIGSR in a cell line (CAC2) from human adenoid cystic carcinoma. Our in vitro results suggest that the complex 67LR—YIGSR regulates morphology of CAC2 cells. Furthermore, this complex peptide-receptor is involved in epithelial—mesenchymal transition, modulation of β-catenin expression and increase in the migration of CAC2 cells.

In 1983, three independent laboratories reported the purification of a 67 kDa molecule, designated the 67 kDa laminin receptor (67LR).[32—34] Different studies to elucidate the role played by the 67LR on tumour progression have shown an increase in 67LR expression in tumours compared with normal tissues and a correlation between 67LR expression, invasive phenotype of the tumour and poor prognosis.[18,20,35,36] Despite being one of the best-characterised members of the laminin receptor with a number of potential functions, the precise structure of this molecule has not been elucidated. A cDNA encoding a cytoplasmic precursor of 37 kDa has been identified, which shows intriguing identity to the ribosomal protein-associated protein p40.[18,37—40] This highly conserved multifunctional 37 kDa protein is considered to be the precursor of the 67 kDa laminin receptor, but the exact mechanism by which this occurs is not known.[37—39,41] The mature form of 67 kDa laminin receptor has been show to physically associate with α6β4 integrin during laminin recognition.[42]

Adenoid cystic carcinoma expressed the 67LR receptor. This expression was observed in all subtypes studied, but it was more prominent in cribriform and solid patterns. This finding demonstrates the value of this receptor as a prognostic marker, since adenoid cystic carcinoma with solid subtype shows a poorer prognosis compared to others patterns.[1,2,43] Moreover, the prominent expression of 67LR in the solid subtype confirms that the presence of this molecule correlates with malignancy.[18,20,21,35,36,44] This receptor was also expressed in CAC2 cells. Our results with immunofluorescence suggested that the expression of 67LR in these cells was similar to that observed in other cell lines.[26,27,42,45]

The 67LR exhibits high affinity for laminin and binds a particular laminin-derived peptide, the sequence YIGSR, located at the short arm of the β1 chain.[17] This peptide is involved in cell adhesion and migration.[17,46—48] It is important to emphasise that the laminin sequence is composed by different bioactive peptides, with relevant functions, such as adhesion, proliferation, migration, cellular growth, angiogenesis and protease activity.[11,12,47,49—51] We have already demonstrated that the sequence SIKVAV, located at the carboxi terminus of laminin α chain regulates morphology and protease activity of CAC2 cells.[11,12] We are currently studying the role played by YIGSR and its ligand, the 67LR, in these cells.

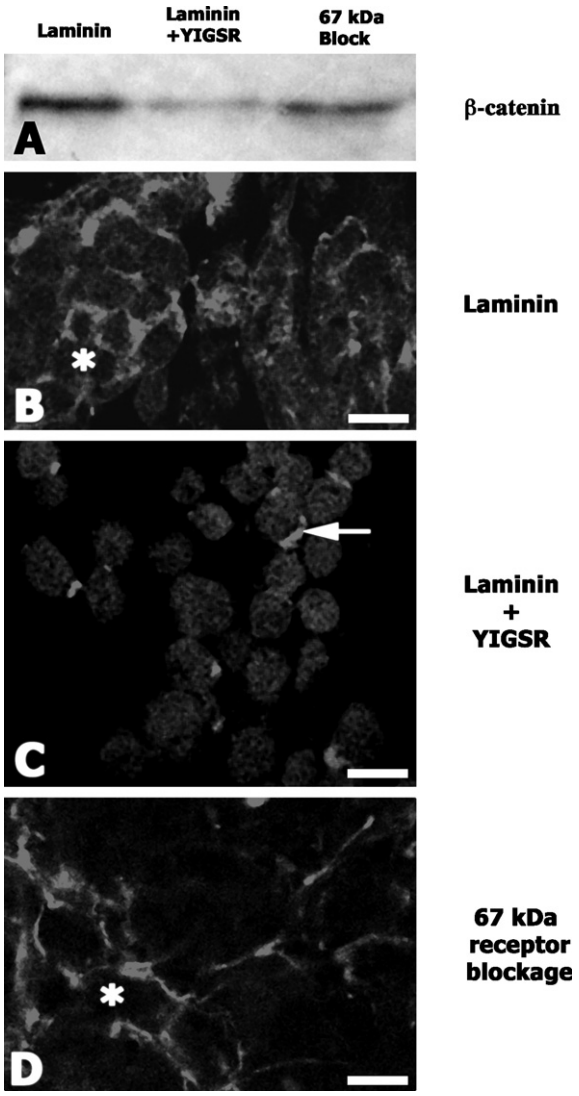

Figure 4 Three-dimensional cultures of CAC2 cells embedded into either laminin-111 gel or YIGSR-enriched laminin-111 were subjected to immunoblot. YIGSR decreases the amount of β-catenin in CAC2 cells (A middle lane) compared to cells grown inside laminin-111 gel (A, left lane). Pretreatment of CAC2 cells with anti-67LR restores β-catenin expression (A, right lane). B–D are confocal sections from whole mount preparations labelled to β-catenin. CAC2 cells cultured inside laminin-111 gel exhibit β-catenin located at cell boundaries (B, asterisk). Treatment by YIGSR decreases the presence of this adhesion molecule (C). A discrete label is observed at the cell surface (C, arrow). CAC2 cells were pretreated with antibody against 67LR and grown inside YIGSR-enriched laminin-111 (D). Blockage of 67LR restores β-catenin expression at cell edges (D, asterisk). Scale bar: 20 μm.

Adenoid cystic carcinoma expresses the 67LR in vivo and in vitro. This expression has prompted us to study the effect of the 67LR and its ligand YIGSR on CAC2 cells. The reason for studying YIGSR and to assume that this peptide is present in vivo is based on extracellular matrix biology. For a long time, extracellular matrix was only considered as an architectural support formed by intact molecules. However, this structure is not a mere scaffold for cells but it also harbours cryptic biological functions that can be revealed when intact molecules are fragmented by proteolysis.[52–55] The proteolysis of this matrix can weaken the structural integrity of tissues, stimulate cellular invasion, trigger apoptosis or proliferation, and release matrix bound growth factors.[54] In addition, emerging evidence suggests that proteolytic enzymes process extracellular matrix molecules, releasing fragments and bioactive peptides with biological responses distinct from intact molecules.[54] These fragments and bioactive peptides are likely to be present in basically every extracellular matrix molecule.[54]

Adenoid cystic carcinoma expresses laminin-111 in vivo and in vitro. CAC2 cells expressed significant amount of laminin, characterised by a network distributed throughout the cell membrane. Other studies have previously shown prominent expression of laminin in adenoid cystic carcinoma.[3,4,43,56,57] We may speculate that tumour cells secrete laminin, which is broken down afterwards by different proteases. The outcome of this process is the formation of small laminin fragments, many of them rich in bioactive peptides.[53–55] This was the rationale of our assay with YIGSR-enriched laminin-111, by creating a microenvironment where CAC2 cells are exposed to preparations with high concentrations of this bioactive peptide. By using this system we obtained information on the effect of the peptide in CAC2 cells. Furthermore, by blocking the 67LR with a specific antibody we addressed whether this receptor is involved in any potential effect of YIGSR. This antibody inhibits the attachment of different cell lines to laminin.[26,27] Moreover, we have tested the direct effect of this antibody in the adhesion of CAC2 cells to laminin-111. Adhesion assay confirmed that the 67LR decreased the adhesion of CAC2 cells to this substrate (data not shown). The adhesion decreased but was not abolished, since CAC2 cells still bind laminin, probably through integrins. In our three-dimensional culture the same phenomenon is occurring. CAC2 cells with blocked 67LR still interact with YIGSR-enriched laminin-111, probably through integrins. However, we may assume that the antibody inhibited the signalling process dependent on the 67LR.

Salivary gland neoplasms have been successfully studied through two-dimensional models.[56–58] We have been studying these tumours using three-dimensional culture systems.[7,8,11–13] A number of cell models have been coupled with appropriate three-dimensional matrices and show fruitful results in recapitulating tissue function.[59,60] Extensive studies have been reported for liver, salivary gland, vasculature, bone, lung, skin, intestine, kidney and mammary and thyroid glands.[59–64] Three-dimensional culture has provided valuable insight into the basic molecular mechanisms of polarity, branching morphogenesis, adhesion and differentiation.

CAC2 cells embedded into laminin-111 gel formed closely packed formations of epithelioid cells. We have previously observed this effect in CAC2 cells.[11,12] On the other hand, cells treated by YIGSR-enriched laminin-111 showed a completely different phenotype. Cells grown in these preparations were non-cohesive and spindle-shaped, with morphology similar to fibroblasts. This feature resembled the epithelial–mesenchymal transition, which occurs in different malignant neoplasms.[65]

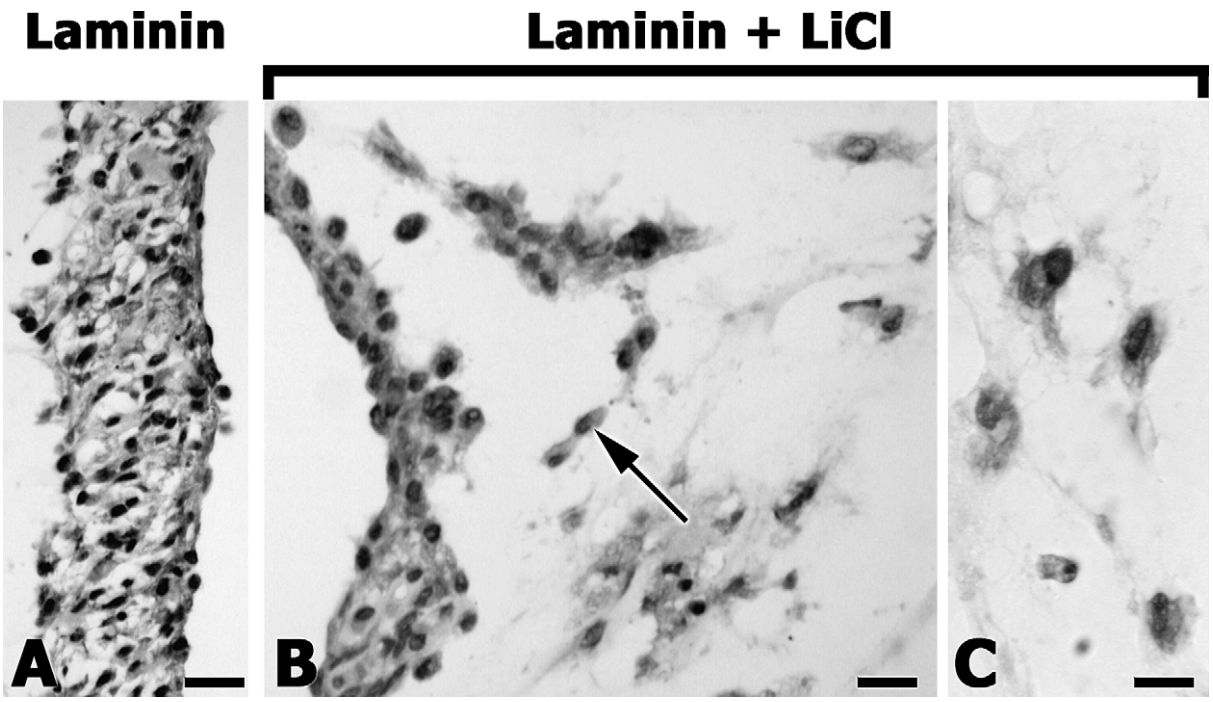

Figure 5 Activation of the Wnt pathway in CAC2 cells through lithium chloride (LiCl). Three-dimensional cultures of CAC2 cells embedded into laminin-111 gel containing 20 μM lithium chloride were prepared and studied by light microscopy. Control cells, grown inside laminin, are closely packed and epithelioid (A). On the other hand, CAC2 cells treated by lithium chloride are mostly round and non-cohesive (B and C). Spindle-shaped and fibroblast-like cells are also observed (B, arrow and C). These results indicate that lithium chloride, an activator of the Wnt signalling pathway, induced CAC2 cells to create a phenotype similar to that observed when cells are treated with YIGSR. Scale bar: (A and B) 20 μm; (C) 10 μm.

Epithelial cells can convert into mesenchymal cells by a process known as the epithelial–mesenchymal transition (EMT).[65,66] The term EMT describes a series of events during which epithelial cells lose many of their epithelial characteristics and take on properties that are typical of mesenchymal cells, which require complex changes in cell architecture and behaviour.[65,66] The transition from epithelial- to mesenchymal-cell characteristics encompasses a spectrum of inter- and intra-cellular changes, not all of which are always seen during EMT.[65,66] EMT does not therefore necessarily refer to a lineage switch. A central feature of EMT is the disassembly of the E-cadherin–β-catenin complex located at the cell membrane.[65] As a result cell–cell dissociation occurs. Furthermore, EMT triggers cell migration, driven by rearrangement of the cytoskeleton and formation of new cell–substratum contacts.[65,66]

CAC2 cells treated by YIGSR showed morphology of cell exhibiting EMT. This result was also dependent on 67LR, since the blockage of this receptor abolished the effect of YIGSR. A key molecule in EMT is β-catenin.[65] We then decided to study whether the 67LR and YIGSR modulate β-catenin expression in these cells. Western blot showed that YIGSR inhibited the expression of β-catenin in CAC2 cells. On the other hand, blockage of the 67LR by antibody restored β-catenin expression. Whole mount immunofluorescence confirmed this result. Thus, we may assume that the 67LR and its ligand YIGSR would regulate the expression of β-catenin in CAC2 cells. Moreover, this evidence also supports a role played by 67LR–YIGSR in the epithelial–mesenchymal transition depicted by CAC2 cells.

The complex 67LR–YIGSR is involved in EMT and in the regulation of β-catenin expression in CAC2 cells. We may speculate that both 67LR and YIGSR would be involved in signalling events related to β-catenin expression. This molecule binds tightly to the cytoplasmic domain of cadherins and links cadherins to the actin cytoskeleton.[67] Cadherins act as a negative regulator of β-catenin signalling as it sequesters this molecule from the nucleus.[67] Disruption of the cadherin–catenin complex at the cell surface release β-catenin in the cytoplasm and makes this molecule available for signalling. The cytoplasmic stabilisation and accumulation of β-catenin is mediated by Wnt.[67,68] Conventional Wnt signalling causes β-catenin accumulation in a complex with the transcription factor TCF/LEF that regulates target gene expression.[67,68]

We also addressed whether CAC2 cells would be influenced by Wnt pathway by treating these cells with lithium chloride (LiCl). Activation of the canonical Wnt signalling pathway can be achieved with LiCl, which has been shown to inhibit glycogen synthase kinase-3 (GSK-3), an enzyme that phosphorylates β-catenin in the cytoplasm, targeting it for ubiquitination and degradation 30. Three-dimensional cultures of CAC2 cells treated by lithium chloride showed morphology of cells undergoing EMT. Phenotypic modifications induced by LiCl are similar to that observed when CAC2 cells are treated with YIGSR. This result suggests that signals generated by YIGSR may be transduced by Wnt pathway in CAC2 cells.

CAC2 cells treated by YIGSR showed signs of disassembly of the E-cadherin–β-catenin complex located at the cell

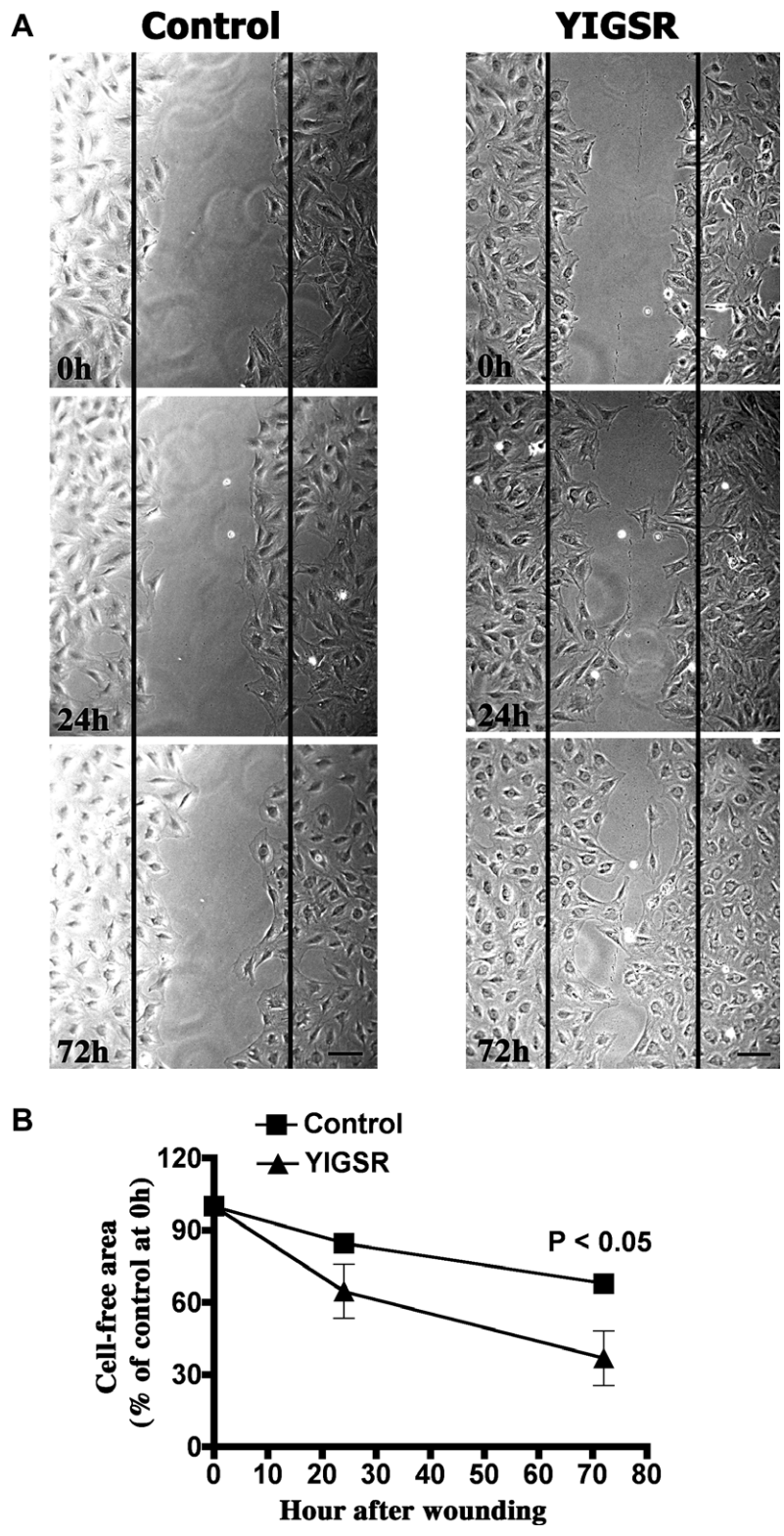

Figure 6 The effect of YIGSR in CAC2 cell migration was examined by using an in vitro wound healing assay. Phase contrast microscopy (A) shows that the cell-free wound gap of CAC2 control monolayer heals slowly. However, in the presence of YIGSR, the closure of the wounded area is significantly accelerated (A). More cells appear in the wounded gap, which represents enhanced migratory activity towards the wounded area (A). In control CAC2 cells, 85% of the wound area remains cell-free at 24 h after wounding (B). However, in cells treated by YIGSR, cell-free area decreases to 65% (B). After 72 h of treatment by YIGSR only 35% of the wound area remains cell-free (B). On the other hand, 70% of cell-free area is observed in control samples (B). Results in B represent mean ± standard error of three experiments carried out at least three times. Statistical analysis was carried out using ANOVA. Scale bar in A: 50 μm.

surface and morphological evidences of cell–cell dissociation. Immunofluorescence showed a discrete expression of β-catenin in CAC2 cells treated by YIGSR. However, Western blot examination of these samples showed that β-catenin is still present, even in smaller amounts. Thus, we may assume that cell–cell dissociation induced by YIGSR resulted in the disassembly of E-cadherin–β-catenin from the plasma membrane. β-catenin is probably released from the cell membrane and accumulated in the cytoplasm of CAC2 cells, triggering different signalling cascades.

CAC2 cells showed signs of epithelial–mesenchymal transition. Another typical feature of EMT, besides disruption of cellular junctions, is the increase in cellular migratory rate.[65,66] Monolayer wound assays showed that YIGSR increase the migration of CAC2 cells compared to controls. This result also support our finding that the complex 67LR–YIGSR is involved in epithelial–mesenchymal transition of CAC2 cells. Our results showing that the peptide YIGSR stimulates migration also confirm previous reports, showing that basement membrane proteins stimulate the migration of adenoid cystic carcinoma cells in vitro.[58]

We may speculate that this migratory effect would influence the behaviour of adenoid cystic carcinoma in vivo. Adenoid cystic carcinoma exhibits affinity for basement membrane-rich tissues, such as nerves and vessels, leading to a perineural spreading and metastasis even long time after treatment.[2,6,43,58] A key step in the metastatic process is the proteolytic degradation of basement membrane components such as proteoglycans, laminin and type IV collagen by proteases secreted by stromal and tumour cells.[16] This proteolytic cleavage not only removes physical barriers but also converts basement membrane components into substrates suitable for migration, presumably by exposure of motility-promoting sites, among them YIGSR.[16] In a cell line derived from human adenoid cystic carcinoma we have shown that this peptide and its receptor 67LR are probably involved in epithelial–mesenchymal transition, modulation of β-catenin expression, and migratory activity. Establishment of the precise molecular pathway involved in the transduction of signals generated by the 67LR and YIGSR would be important to better understand the role played by this receptor in adenoid cystic carcinoma.

Acknowledgement

This work was supported by The State of São Paulo Research Foundation (FAPESP, Grant 00/04693-0). V.M.F. is recipient of a Post Doctoral fellowship from FAPESP (06/54963-0). L.N.G.S. and E.C.O. are graduate students (FAPESP PhD fellowships 05/55602-9 and 05/55601-2, respectively).

References

1. Seifert G, Sobin LH. The World Health Organization's histological classification of salivary gland tumors. A commentary on the second edition. *Cancer* 1992;**70**(2):379–85.
2. Bradley PJ. Adenoid cystic carcinoma of the head and neck: a review. *Curr Opin Otolaryngol Head Neck Surg* 2004;**12**(2):127–32.
3. Cheng J et al. Biosynthesis of basement membrane molecules by salivary adenoid cystic carcinoma cells: an immunofluorescence and confocal microscopic study. *Virchows Arch* 1995;**426**(6):577–86.
4. Cheng J, Saku T, Okabe H, Furthmayr H. Basement membranes in adenoid cystic carcinoma. An immunohistochemical study. *Cancer* 1992;**69**(11):2631–40.
5. Albores-Saavedra J, Wu J, Uribe-Uribe N. The sclerosing variant of adenoid cystic carcinoma: a previously unrecognized neoplasm of major salivary glands. *Ann Diagn Pathol* 2006;**10**(1):1–7.
6. Kumamoto M et al. Expression of laminin 5 basement membrane components in invading and recurring adenoid cystic carcinoma of the head and neck. *Auris Nasus Larynx* 2006;**33**(2):167–72.
7. Capuano AC, Jaeger RG. The effect of laminin and its peptide SIKVAV on a human salivary gland myoepithelioma cell line. *Oral Oncol* 2004;**40**(1):36–42.
8. de Oliveira PT, Jaeger MM, Miyagi SP, Jaeger RG. The effect of a reconstituted basement membrane (matrigel) on a human salivary gland myoepithelioma cell line. *Virchows Arch* 2001;**439**(4):571–8.
9. Franca CM, Jaeger MM, Jaeger RG, Araujo NS. The role of basement membrane proteins on the expression of neural cell adhesion molecule (N-CAM) in an adenoid cystic carcinoma cell line. *Oral Oncol* 2000;**36**(2):248–52.
10. Franca CM, Jaeger RG, Freitas VM, Araujo NS, Jaeger MM. Effect of N-CAM on in vitro invasion of human adenoid cystic carcinoma cells. *Oral Oncol* 2001;**37**(8):638–42.
11. Freitas VM, Jaeger RG. The effect of laminin and its peptide SIKVAV on a human salivary gland adenoid cystic carcinoma cell line. *Virchows Arch* 2002;**441**(6):569–76.
12. Freitas VM, Scheremeta B, Hoffman MP, Jaeger RG. Laminin-1 and SIKVAV a laminin-1-derived peptide, regulate the morphology and protease activity of a human salivary gland adenoid cystic carcinoma cell line. *Oral Oncol* 2004;**40**(5):483–9.
13. Jaeger MM, Araujo VC, Kachar B, Jaeger RG. Effect of spatial arrangement of the basement membrane on cultured pleomorphic adenoma cells. Study by immunocytochemistry and electron and confocal microscopy. *Virchows Arch* 1997;**430**(6):467–77.
14. Jaeger RG, de Oliveira PT, Jaeger MM, de Araujo VC. Expression of smooth-muscle actin in cultured cells from human plasmacytoid myoepithelioma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1997;**84**(6):663–7.
15. Loducca SV, Raitz R, Araujo NS, Araujo VC. Polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma: distinct architectural composition revealed by collagen IV, laminin and their integrin ligands (alpha2beta1 and alpha3beta1). *Histopathology* 2000;**37**(2):118–23.
16. Ardini E et al. Identification of a novel function for 67-kDa laminin receptor: increase in laminin degradation rate and release of motility fragments. *Cancer Res* 2002;**62**(5):1321–5.
17. Graf J et al. A pentapeptide from the laminin B1 chain mediates cell adhesion and binds the 67,000 laminin receptor. *Biochemistry* 1987;**26**(22):6896–900.
18. Boukerche H, Su ZZ, Kang DC, Fisher PB. Identification and cloning of genes displaying elevated expression as a consequence of metastatic progression in human melanoma cells by rapid subtraction hybridization. *Gene* 2004;**343**(1):191–201.
19. Givant-Horwitz V et al. Expression of the 67 kDa laminin receptor and the alpha6 integrin subunit in serous ovarian carcinoma. *Clin Exp Metast* 2003;**20**(7):599–609.
20. Menard S, Tagliabue E, Colnaghi MI. The 67 kDa laminin receptor as a prognostic factor in human cancer. *Breast Cancer Res Treat* 1998;**52**(1–3):137–45.
21. Pelosi G et al. High-affinity monomeric 67-kD laminin receptors and prognosis in pancreatic endocrine tumours. *J Pathol* 1997;**183**(1):62–9.

22. Zhou L, Xie M, Zhou JQ, Tao L. 67-kDa laminin receptor in human laryngeal squamous cell carcinoma. *Laryngoscope* 2006;**116**(1):28—32.
23. Berno V et al. The 67 kDa laminin receptor increases tumor aggressiveness by remodeling laminin-1. *Endocr Relat Cancer* 2005;**12**(2):393—406.
24. Fulop T, Larbi A. Putative role of 67 kDa elastin-laminin receptor in tumor invasion. *Semin Cancer Biol* 2002;**12**(3): 219—29.
25. Aumailley M et al. A simplified laminin nomenclature. *Matrix Biol* 2005;**24**(5):326—32.
26. Clement B, Segui-Real B, Savagner P, Kleinman HK, Yamada Y. Hepatocyte attachment to laminin is mediated through multiple receptors. *J Cell Biol* 1990;**110**(1):185—92.
27. Mercurio AM, Shaw LM. Macrophage interactions with laminin: PMA selectively induces the adherence and spreading of mouse macrophages on a laminin substratum. *J Cell Biol* 1988;**107**(5):1873—80.
28. Weaver VM et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* 1997;**137**(1):231—45.
29. Du Q et al. Regulation of human nitric oxide synthase 2 expression by Wnt beta-catenin signaling. *Cancer Res* 2006;**66**(14):7024—31.
30. Clement-Lacroix P et al. Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proc Natl Acad Sci USA* 2005;**102**(48):17406—11.
31. Lee H, Goetzl EJ, An S. Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am J Physiol Cell Physiol* 2000;**278**(3):C612—8.
32. Rao NC, Barsky SH, Terranova VP, Liotta LA. Isolation of a tumor cell laminin receptor. *Biochem Biophys Res Commun* 1983;**111**(3):804—8.
33. Malinoff HL, Wicha MS. Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. *J Cell Biol* 1983;**96**(5):1475—9.
34. Lesot H, Kuhl U, Mark KV. Isolation of a laminin-binding protein from muscle cell membranes. *Embo J* 1983;**2**(6):861—5.
35. Montuori N, Sobel ME. The 67-kDa laminin receptor and tumor progression. *Curr Top Microbiol Immunol* 1996;**213**(Pt 1): 205—14.
36. Sobel ME. Differential expression of the 67 kDa laminin receptor in cancer. *Semin Cancer Biol* 1993;**4**(5):311—7.
37. Ardini E et al. The 67-kDa laminin receptor originated from a ribosomal protein that acquired a dual function during evolution. *Mol Biol Evol* 1998;**15**(8):1017—25.
38. Castronovo V et al. Biosynthesis of the 67 kDa high affinity laminin receptor. *Biochem Biophys Res Commun* 1991;**177**(1): 177—83.
39. Castronovo V, Taraboletti G, Sobel ME. Functional domains of the 67-kDa laminin receptor precursor. *J Biol Chem* 1991;**266**(30):20440—6.
40. Wewer UM et al. Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. *Proc Natl Acad Sci USA* 1986;**83**(19):7137—41.
41. Buto S et al. Formation of the 67-kDa laminin receptor by acylation of the precursor. *J Cell Biochem* 1998;**69**(3):244—51.
42. Ardini E et al. Co-regulation and physical association of the 67-kDa monomeric laminin receptor and the alpha6beta4 integrin. *J Biol Chem* 1997;**272**(4):2342—5.
43. Shintani S, Alcalde RE, Matsumura T, Terakado N. Extracellular matrices expression in invasion area of adenoid cystic carcinoma of salivary glands. *Cancer Lett* 1997;**116**(1):9—14.
44. Magnifico A et al. Peptide G, containing the binding site of the 67-kDa laminin receptor, increases and stabilizes laminin binding to cancer cells. *J Biol Chem* 1996;**271**(49):31179—84.
45. Karpatova M et al. Shedding of the 67-kD laminin receptor by human cancer cells. *J Cell Biochem* 1996;**60**(2):226—34.
46. Graf J et al. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell* 1987;**48**(6):989—96.
47. Iwamoto Y et al. Synthetic pentapeptide from the B1 chain of laminin promotes B16F10 melanoma cell migration. *J Cell Physiol* 1988;**134**(2):287—91.
48. Iwamoto Y et al. YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation. *Science* 1987;**238**(4830):1132—4.
49. Nomizu M et al. Cell binding sequences in mouse laminin alpha1 chain. *J Biol Chem* 1998;**273**(49):32491—9.
50. Nomizu M et al. Cell adhesive sequences in mouse laminin beta1 chain. *Arch Biochem Biophys* 2000;**378**(2):311—20.
51. Nomizu M et al. Identification of cell binding sequences in mouse laminin gamma1 chain by systematic peptide screening. *J Biol Chem* 1997;**272**(51):32198—205.
52. Faisal Khan KM, Laurie GW, McCaffrey TA, Falcone DJ. Exposure of cryptic domains in the alpha 1-chain of laminin-1 by elastase stimulates macrophages urokinase and matrix metalloproteinase-9 expression. *J Biol Chem* 2002;**277**(16):13778—86.
53. Ghosh S, Stack MS. Proteolytic modification of laminins: functional consequences. *Microsc Res Tech* 2000;**51**(3):238—46.
54. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. *Curr Opin Cell Biol* 2004;**16**(5):558—64.
55. Schenk S, Quaranta V. Tales from the crypt[ic] sites of the extracellular matrix. *Trends Cell Biol* 2003;**13**(7):366—75.
56. Shirasuna K et al. Extracellular matrix production and degradation by adenoid cystic carcinoma cells: participation of plasminogen activator and its inhibitor in matrix degradation. *Cancer Res* 1993;**53**(1):147—52.
57. Shirasuna K et al. Biological characterization of pseudocyst-forming cell lines from human adenoid cystic carcinomas of minor salivary gland origin. *Cancer Res* 1990;**50**(13):4139—45.
58. Abu-Ali S et al. Expression of the urokinase receptor regulates focal adhesion assembly and cell migration in adenoid cystic carcinoma cells. *J Cell Physiol* 2005;**203**(2):410—9.
59. Nelson CM, Bissell MJ. Modeling dynamic reciprocity: engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation. *Semin Cancer Biol* 2005;**15**(5):342—52.
60. Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci* 2003;**116** (Pt 12):2377—88.
61. Azuma M, Sato M. Morphogenesis of normal human salivary gland cells in vitro. *Histol Histopathol* 1994;**9**(4):781—90.
62. Azuma M, Tamatani T, Fukui K, Bando T, Sato M. Enhanced proteolytic activity is responsible for the aberrant morphogenetic development of SV40-immortalized normal human salivary gland cells grown on basement membrane components. *Lab Invest* 1994;**70**(2):217—27.
63. Burford-Mason AP, Dardick I, MacKay A. Collagen gel cultures of normal salivary gland: conditions for continued proliferation and maintenance of major cell phenotypes in vitro. *Laryngoscope* 1994;**104**(3 Pt 1):335—40.
64. Yang J, Larson L, Flynn D, Elias J, Nandi S. Serum-free primary culture of human normal mammary epithelial cells in collagen gel matrix. *Cell Biol Int Rep* 1982;**6**(10):969—75.
65. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial—mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006;**172**(7):973—81.
66. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial—mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006;**7**(2): 131—42.
67. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004;**303**(5663):1483—7.
68. Moon RT, Bowerman B, Boutros M, Perrimon N. The promise and perils of Wnt signaling through beta-catenin. *Science* 2002;**296**(5573):1644—6.