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Research Article

Collagen XVII promotes integrin-mediated squamous cell carcinoma transmigration—A novel role for αII_b integrin and tirofiban

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ABSTRACT

The expression of collagen XVII (BP180), a transmembrane hemidesmosomal component, is upregulated in invasive areas of epithelial tumors. The collagenous ectodomain of collagen XVII is cleaved from the plasma membrane of keratinocytes and malignant epithelial cells. The released ectodomain is predicted to regulate cell detachment, differentiation, and motility. We report that the cell adhesion domain of collagen XVII, Col15, is able to chemotactically attract invasive HSC-3 SCC cells but not keratinocytes. Analysis of integrin expression revealed that HSC-3 cells, unlike keratinocytes, express αII_b integrin, a platelet-specific fibrinogen receptor. We show that this novel chemotactic function is mediated by the known Col15-binding integrins $\alpha 5\beta 1$ and αv and the platelet integrin αII_b. Moreover, we report that tirofiban, a FDA-proved αII_b integrin-blocking drug widely used for the therapy of acute coronary ischaemic syndrome and the prevention of thrombotic complications, inhibits the Col15 chemotaxis of HSC-3 carcinoma cells. Together, these data demonstrate a novel interaction between collagen XVII and αII_b integrin and also suggest a possibility to use tirofiban to inhibit the invasion and progression of αII_b expressing SCC tumors.

Introduction

Collagen XVII (or BP180) is a structural component of hemidesmosomes, which connect the intracellular space of epithelial cells, such as basal keratinocytes in skin or mucosa, to the underlying basement membrane. This transmembrane collagen consists of a globular intracellular domain, a short transmembrane stretch, and a collagenous ectodomain, which consists of 15 collagenous subdomains. The intracellular domain interacts with ß4 integrin, plectin, and BP230, and

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the extracellular domain binds to α6 integrin and laminin-5 [1–3]. The ectodomain is constitutively shed from the cell surface by three members of ADAMs (a disintegrin and metalloproteinase) and can be extracted in vivo from epidermis, amniotic fluid, and SCC tissue [1–5].

It is well established that collagen XVII is associated with both inherited and acquired blistering skin diseases [1–3]. However, the functions of collagen XVII and its shedding in keratinocyte adhesion and migration in various physiological and pathological situations are not yet fully understood. The increased shedding of the collagen XVII ectodomain by transfection or the addition of purified ectodomain to cell culture media inhibits the migration of keratinocytes [5]. Accordingly, the absence of collagen XVII in mutated keratinocytes increases keratinocyte migration [6]. In contrast, the largest collagenous domain of collagen XVII, Col15, promotes epithelial adhesion and spreading via the α5β1 and αv integrins, which are not expressed on quiescent keratinocytes, but are expressed on migrating wound keratinocytes and invasive carcinoma cells [7–9]. We have previously shown, that Col15 domain contains 12 KGD motifs, which are recognized by αv and α5β1 integrins [9]. These integrins mediate low affinity binding to KGD, while they have stronger interaction with RGD sites in other ligands. Platelet integrin $\alpha II_b\beta 3$ is reported to recognize the KDG motif with higher affinity [10]. Taken together, the function of collagen XVII in keratinocyte adhesion and migration seems to vary, depending on the spatial or temporal biological context.

Collagen XVII may also play a role in tumor progression, since in invasive areas of epithelial tumors, collagen XVII as well as its ligands α6β4 integrin and laminin-5 are upregulated [4,11,12]. In the present study, the potential association of the cell adhesion domain Col15 of collagen XVII with malignant migration was explored. The results revealed a promigratory function for the Col15 domain in carcinoma cell transmigration. Experiments with function-blocking anti-integrin antibodies indicated that the α5β1, αv, and αII_b integrins mediate the migration-promoting effect of Col15. Furthermore, tumor cells that express $\alpha II_b\beta 3$ integrin may use this fibrinogen receptor in binding to collagen XVII. This mechanism represents a novel receptor–ligand interaction and may explain why some malignant cells, such as SCC and melanoma cells, express this platelet-specific integrin.

Materials and methods

Tissue samples and cells

For healthy volunteers (age: 22–28 years; $n = 5$), 3 mm full-thickness punch wounds were generated on the buccal mucosa. The wounds were allowed to heal for 3 days, after which the wound area was biopsied using a 6-mm punch. The tissue samples were fixed with 4% formalin and embedded in paraffin. Samples of tongue SCC tumors ($n = 35$) were obtained from the Department of Pathology, Oulu University Hospital. The study was performed with the approval of the Ethical Committee of the University of Oulu.

The highly invasive human tongue SCC cell line HSC-3 (Japan Health Science Research Resources Bank, JRCB 0623) was cultured in 1:1 DMEM/Ham's Nutrient Mixture F-12 (Gibco BRL; Grand Island, NY) containing 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin-B, and 0.4 μg/ml hydrocortisone. HaCaT keratinocytes, a kind gift of Dr. N. Fusening (German Cancer Research Center, Heidelberg, Germany), were grown in serum-free keratinocyte medium (Gibco BRL; Grand Island, NY) supplemented with bovine pituitary extract, recombinant epidermal growth factor, 100 IU/ml penicillin, and 2.5 μg/ml amphotericin-B.

Antibodies and immunostaining

Collagen XVII immunostainings were carried out using a polyclonal NC16A antibody [5]. The polyclonal laminin-5 antibody recognizing the γ2 chain was a generous gift from Prof. Karl Tryggvason [11]. Flow cytometry analysis was performed with antibodies against the α5 (mAb 16) [13], αv (mAb L-230), αII_b (CD41a, BD Pharmingen; Franklin Lakes, NJ, USA), and β1 (Santa Cruz Biotechnology, Santa Cruz, CA) integrins. FITC-conjugated anti-rat and anti-mouse secondary antibodies were from DAKO A/S (Clostrub, Denmark). Function-blocking α5 (mAb 16) [13], αv (mAb L-230), β1 (R-322, rabbit polyclonal) [14], and αII_b (CD41a, BD Pharmingen; Franklin Lakes, NJ, USA) integrin antibodies and tirofiban (Aggrastat®, Merck; Hoddesdon, UK) were used in cell transmigration experiments.

Immunoperoxidase staining of collagen XVII and the laminin-5 γ2 chain was performed as previously described [15].

Flow cytometry analysis

HSC-3 or HaCaT cells were incubated with α5 (1:20), β1 (1:20), αv (1:50), or αII_b (1:50) integrin antibodies for 30 min at +4°C and treated with FITC-conjugated anti-rat (α5) (1:100) or anti-mouse (1:20) (αv, αII_b, and β1) secondary antibody for 30 min at +4°C. The cells were then analyzed by flow cytometry.

Immunoprecipitation

A monoclonal antibody against integrin αv (L230) was used in immunoprecipitation assay. Cell cultures were metabolically labeled with 100 μCi/ml of [^{35}S]methionine (Tran^{35}S-label, ICN Biomedicals Inc., Irvine, CA) for 18 h in methionine-free minimum essential medium. Cell monolayers were rinsed on ice with a solution containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM Tris–HCl (pH 7.4) and then detached by scraping. Cell pellets obtained by centrifugation at 500 × g for 5 min were solubilized in 200 μl of the same buffer containing 100 mM n-octyl-β-D-glucopyranoside (Sigma) on ice with occasional vortexing. Insoluble material was removed by centrifugation at 10,000 × g for 5 min at 4°C. Radioactivity in cell lysates was counted, and an equal amount of radioactivity was used in immunoprecipitation assays. Triton X-100 (0.5% v/v) and bovine serum albumin (0.5 mg/ml) were added to the supernatants, which were then precleared by incubation with 50 μl of packed Protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Supernatants were immunoprecipitated with anti-

integrin antibodies for 12 h at 4°C. Immune complexes were recovered by binding to Protein A-Sepharose and washing the beads four times with 25 mM Tris-buffered isotonic saline (pH 7.4) containing 0.5% Triton X-100 and 1 mg/ml bovine serum albumin and twice with 0.5 M NaCl and 25 mM Tris–HCl (pH 7.4). The immunoprecipitates were analyzed by electrophoresis on sodium dodecyl sulfate-containing 6% polyacrylamide gels under nonreducing conditions followed by fluorography.

Production and purification of recombinant Col15

Recombinant production and purification of Col15, the largest collagenous domain of collagen XVII, was carried out as described earlier [16].

Transmigration assay

The undersides of Transwell chamber filters (8.0 μm pore size, Corning, New York) were coated with either 10 μg/ml human plasma fibronectin (Invitrogen, Frederick, Maryland) or 7.5 μg/ml Col15 in PBS for 1 h at RT. After washes in PBS, the filters were blocked with 1% denatured BSA for 30 min at RT. The filters were washed, and the cells were plated at a density of 1.5×10^5 cells/filter in serum-free culture medium. Before plating, the cells were preincubated with antibodies (20–60 μg/ml), tirofiban (0.25–2.5 μg/ml), or vehicle for 30 min at +37°C. Col15 was denatured by heating for 20 min at 56°C. Antibodies were, in all cases, added into both chambers, whereas Col15 was present in the lower chamber, the upper chamber, or both. Cells were allowed to migrate for 6 h at +37°C, after which they were fixed with 10% TCA. The cells that had not migrated to the lower side of the filter were removed with a cotton swab from the upper side. The cells on the underside were then stained with crystal violet and counted in three fields with a 10× objective. The data are expressed as the means ± S.E. of at least three parallel experiments. Statistical analysis was performed using Student's *t* test and statistical significance was set at $P < 0.05$.

Results

Collagen XVII and laminin-5 are coexpressed in invading carcinoma cells, but not in leading keratinocytes during wound healing

The transmembrane collagen XVII and its extracellular ligand, laminin-5, are overexpressed in tumor cells in invasive areas of oral squamous cell carcinomas (SCC) [4,17]. To compare the expression of these proteins during physiological and malignant epithelial migration, parallel tissue sections from experimental punch wounds of oral mucosa and resected sections of oral SCCs were immunostained with antibodies against collagen XVII and laminin-5 γ2 chain. During wound healing, collagen XVII was present on the so-called following cells, the basal keratinocytes of the epithelial outgrowth that are distal to the leading edge cells. The positive staining was located on cell membranes and in the basement membrane (BM) area underlying the cells (Fig. 1A). The leading keratinocytes were negative for collagen XVII (Fig. 1A), whereas laminin-5 γ2

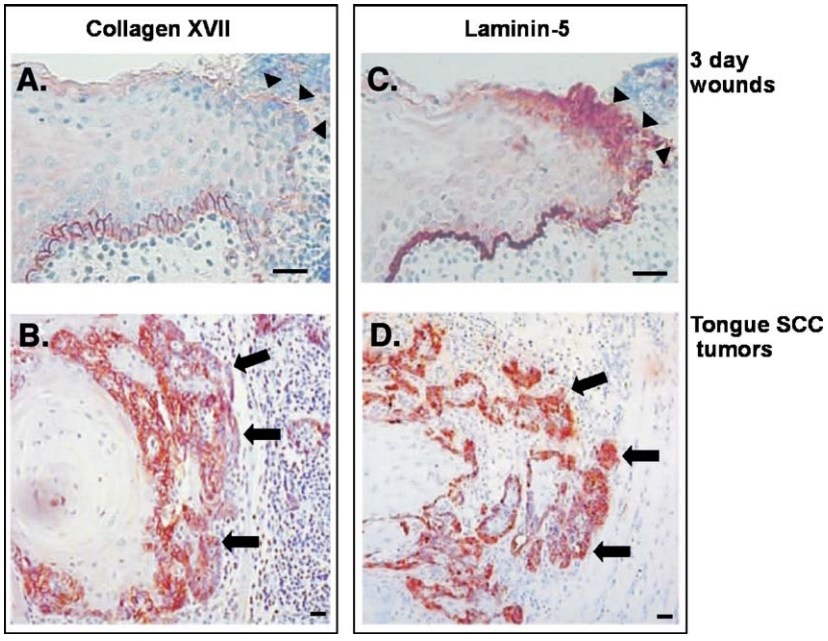

Fig. 1 – Immunostainings were carried out using a polyclonal antibody recognizing the juxtamembranous NC16A domain of collagen XVII and a polyclonal laminin-5 antibody against the domain III of the γ2 chain. In 2 d mucosal wounds, the collagen XVII immunoreaction is negative in the leading keratinocytes (A) (arrowheads), whereas strong cytoplasmic staining of the γ2 chain is seen in the same area (C) (arrowheads). Collagen XVII immunostaining localizes to the cell membranes of the so-called following keratinocyte distal to from the leading edge, whereas laminin-5 is detected in the basement membrane underneath the same cells. Both collagen XVII and laminin-5 are intensely expressed by carcinoma cells in the invading areas of SCC tumors (B, D) (arrows). Scale bars, 20 μm.

chain was visible as strong cytoplasmic staining in the leading keratinocytes and as a continuous band-like signal in the BM underlying the following keratinocytes (Fig. 1C). Both collagen XVII and laminin-5 were upregulated in the invading carcinoma cells of oral SCCs (Figs. 1B, D). These findings indicate that collagen XVII expression, unlike laminin-5, may be differentially regulated in the leading migratory cells during wound healing and carcinoma invasion.

The cell adhesion domain of collagen XVII, COL15, promotes transmigration of carcinoma cells

To assess the influence of collagen XVII on epithelial migration, the transmigration of immortalized keratinocytes, HaCaT cells, and invasive SCC cells (HSC-3) through fibronectin-coated membrane was analyzed in the presence of Col15, the cell adhesion domain of collagen XVII. Addition of

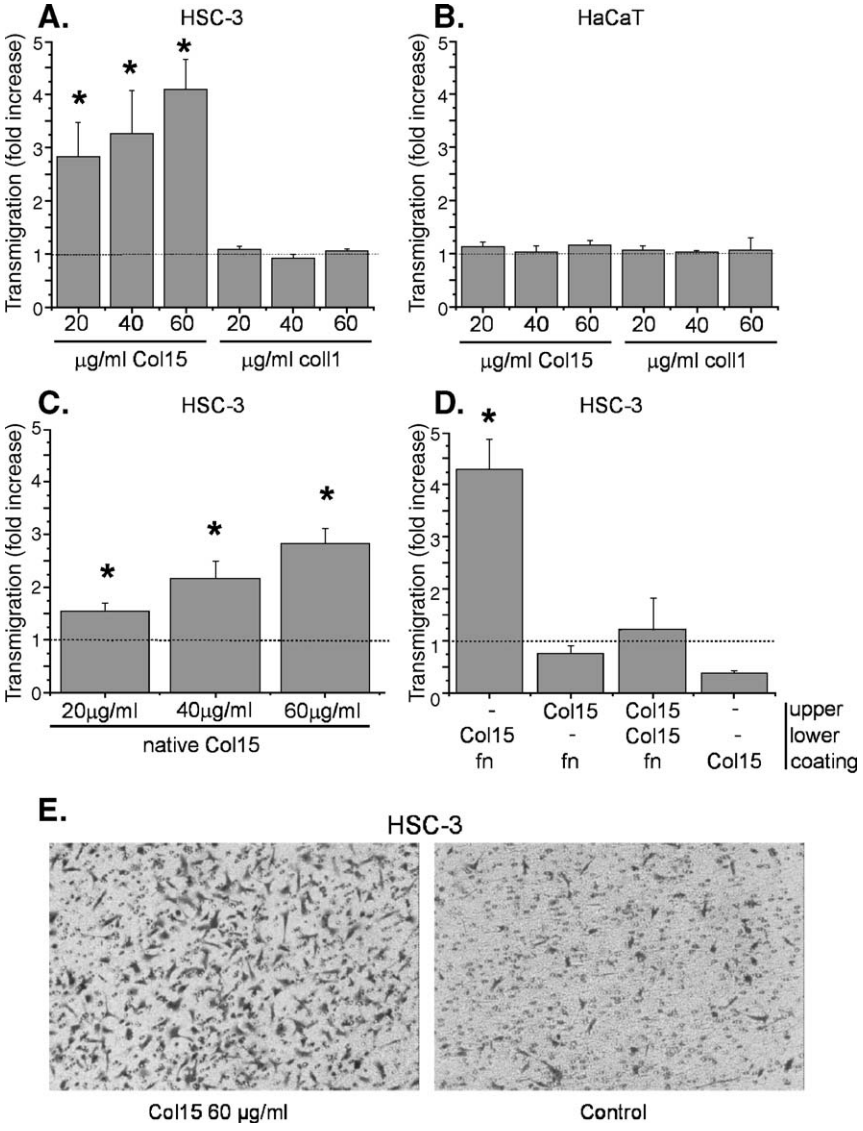

Fig. 2 – In transmigration assays, Transwell filters were coated with either fibronectin (fn) (A–E) or denatured Col15 (D). HSC-3 carcinoma cells or HaCaT cells were plated at a density of 1.5×10^5 cells/well. To study the effect of Col15 on the transmigration of HSC-3 and HaCaT keratinocytes, Col15 was introduced to the cells in a solid (coating of the membrane) or soluble (in the culture media) form and either with (added to either the upper or the lower chamber) or without (added to both chambers) a gradient. Cells were allowed to migrate for 6 h, fixed, stained with crystal violet, and counted. (A) Transmigration of HSC-3 cells was stimulated by the addition of denatured Col15 into the lower chamber (4.3-fold), but not by denatured type I collagen (coll1). Dotted line: transmigration level without any additives. (B) Transmigration of HaCaT keratinocytes is not affected by denatured Col15 or denatured type I collagen (coll1) in the lower chamber. (C) Transmigration of HSC-3 cells is induced up to 2.8-fold in the presence of native Col15 in the lower chamber. (D) Addition of 60 µg/ml denatured Col15 into the upper chamber or both chambers, or coating of the filter with denatured Col15 does not significantly stimulate transmigration of HSC-3 cells. (E) Micrographs of stained HSC-3 cells on the lower side of the Transwell filters. Left: 60 µg/ml denatured Col15 added into the lower chamber. Right: Control (vehicle). The data shown are the means ± S.E.M. of at least three parallel experiments. Statistical analysis was performed using Student's *t* test, Statistical significance is set at $P \leq 0.05$ and indicated by (*).

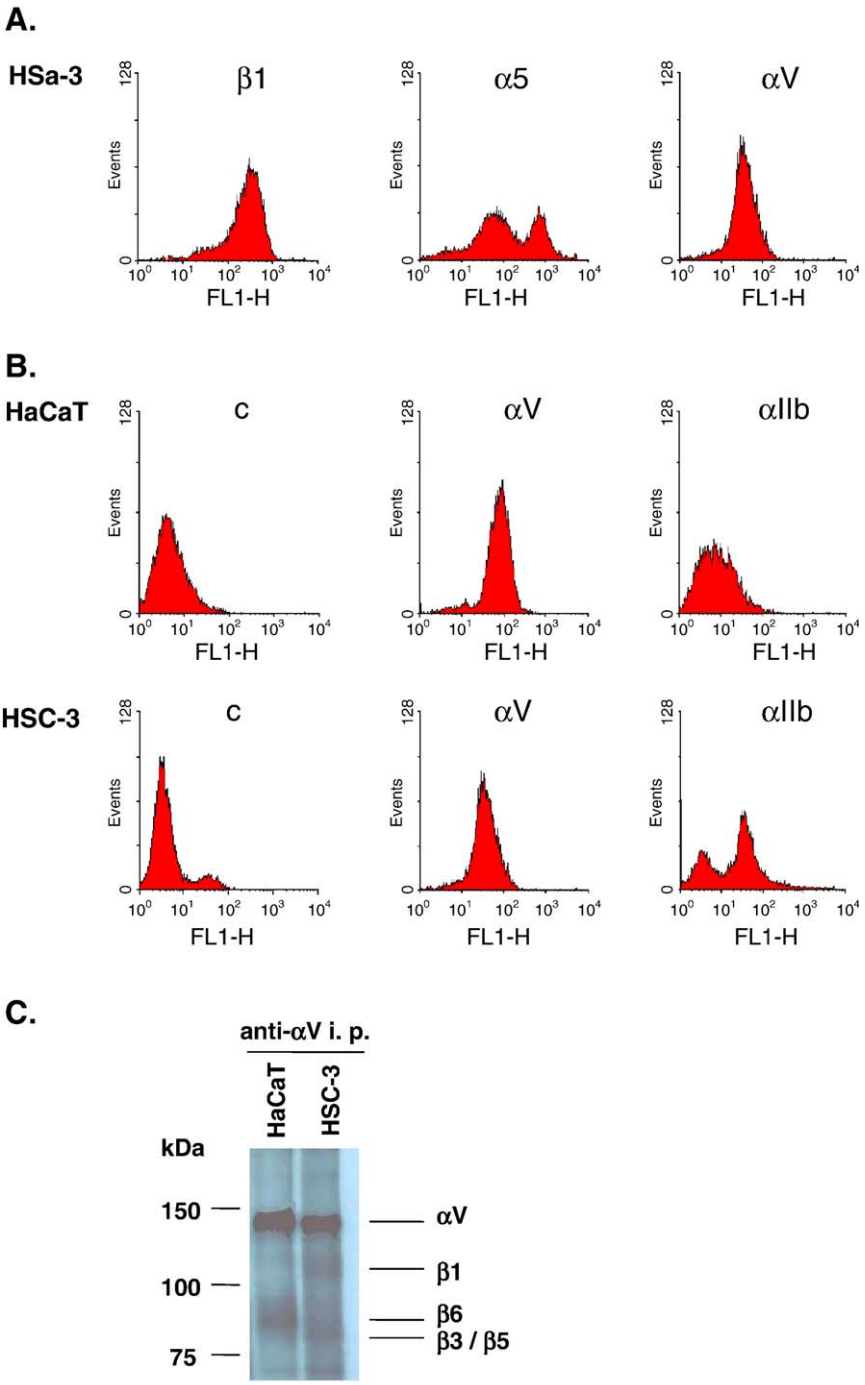

Fig. 3 – To determine the surface expression of integrin subunits in HSC-3 carcinoma cells, cell suspensions were incubated with 1:20 diluted α5 and β1 integrin antibodies and with 1:50 diluted αv integrin antibody (A). To determine the surface expression of integrin subunits in HaCaT and HSC-3 cells, cell suspensions were incubated with 1:50 diluted αv and aIIb antibodies (B). For detection, the cells were then incubated with FITC-conjugated anti-rat (1:100) (α5) or anti-mouse (1:20) (αv, aIIb, and β1) secondary antibody and analyzed by flow cytometry. (FL1-H = channel for measuring the level of FITC-labeling; c = control level, i.e., fluorescence without primary antibody). (C) To analyze the expression of αv integrin and the presence of its coprecipitating β partners an immunoprecipitation assay was performed with specific antibodies against αv subunit. HaCaT and HSC-3 cells were incubated for 18 h with [^{35}S]methionine. Cellular αv integrins were precipitated with L230 antibody and analyzed with electrophoresis and fluorography.

denatured recombinant Col15 into the lower Transwell chamber resulted in up to 4.3-fold concentration-dependent increase ($P \leq 0.05$) in the transmigration of HSC-3 cells (Figs. 2A, D and E), meanwhile Col15, surprisingly, had no effect on the transmigration of HaCaT keratinocytes (Fig. 2B). The presence of Col15 in the upper chamber or the addition of Col15 into both chambers, i.e. without a gradient, did not significantly influence the transmigration level (Fig. 2D). Native Col15 also stimulated HSC-3 transmigration in a concentration-dependent manner (up to 2.8-fold, $P \leq 0.05$), but less efficiently than denatured Col15 (Fig. 2C).

Squamous cell carcinoma cells, but not HaCaT keratinocytes, express a platelet-specific αII$_b$ integrin

Col15 is known to interact with the α5β1 and αv integrins [9]. To further explore the altered response of HSC-3 cells to Col15, the expression of Col15-binding integrin receptors in these cells was determined. Flow cytometry analysis using specific integrin antibodies revealed surface expression of the α5, αv, and β1 integrin subunits in both HSC-3 and HaCaT cells (Figs. 3A, B). HaCaT cells express very low levels of α5β1 integrin [18], whereas an extremely high expression level was detected in a subpopulation of HSC-3 cells (Fig. 3A). Interestingly, αII$_b$ integrin, known as platelet-specific fibrinogen receptor, was expressed on the surface of HSC-3-cells but not in HaCaT cells (Fig. 3B). While most HSC-3 cells were αII$_b$ positive, a smaller subpopulation did not express this integrin. Similarly the analysis with anti-α5 integrin antibody unveiled two populations of HSC-3 cells with different expression levels.

The expression of integrin αv in HSC-3 and HaCaT cells was further studied by immunoprecipitation of metabolically labeled cellular proteins. In accordance with the flow cytometric analysis αv integrin was expressed in both cell lines (Fig. 3C). In the immunoprecipitation assays the β partners of αv subunit could also be studied, since they are known to have small differences in their electrophoretic mobility [19]. As reported earlier, in HaCaT cells the most prominent αv containing heterodimer is αvβ6 and the cells seemed to express some αvβ1 integrin [18] (Fig. 3C). In HSC-3 cells the most abundant β partner of αv integrin seemed to be β1 (Fig. 3C). An antibody against αv integrin could also coprecipitate in HSC-3 cells a β subunit with a molecular mass corresponding to β5 or β3 subunit [19] (Fig. 3C). In separate immunoprecipitation experiments with specific anti-β1 integrin antibodies the presence of this subunit was confirmed in both cell lines, but HSC-3 cells expressed more metabolically labeled β1 integrin than HaCaT cells.

Col15-induced transmigration of carcinoma cells is mediated by αv, α5, and αII$_b$ integrins and inhibited by tirofiban, a snake venom disintegrin

Next, we investigated whether the promigratory effect of Col15 on HSC-3 carcinoma cells can be inhibited by function-blocking antibodies to α5, αv, αII$_b$ or β1 integrins. The anti-β1 integrin antibody completely blocked the transmigration of HSC-3 cells, regardless of whether Col15 was present or absent (not shown). Anti-α5 or anti-αv alone did not inhibit transmigration of HSC-3 cells in the absence of Col15 (Fig. 4). However,

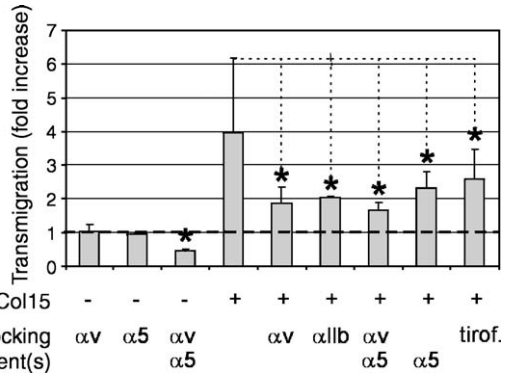

Fig. 4 – In transmigration experiments with function-blocking integrin antibodies (60 μg/ml) and tirofiban (2.5 μg/ml), HSC-3 carcinoma cells were first preincubated with the antibodies or tirofiban for 30 min and plated onto fibronectin-coated Transwell filters. In each experiment, the antibodies were added into both chambers and denatured Col15 into the lower chamber. The cells were then allowed to migrate for 6 h, fixed, stained, and counted. Anti-α5 or anti-αv alone had no effect on the transmigration of HSC-3 cells. However, a combination of the α5 and αv antibodies caused 54% inhibition in the transmigration level. Anti-αv inhibited Col15-induced migration by 53% and anti-α5 antibody by 49%. Together, anti-α5 and anti-αv inhibited Col15-induced migration by 58%. αIIb-integrin antibody (CD41a) inhibited Col15-induced transmigration by 41% and tirofiban, an αIIb-integrin-blocking drug, by 35%. The data shown are the means ± S.E.M. of at least three parallel experiments. Statistical analysis was done using Student's *t* test, Statistical significance is set at $P \leq 0.05$ and indicated by (*).

the combination of anti-α5 and anti-αv antibodies caused a 54% inhibition ($P \leq 0.05$) in the transmigration level (Fig. 4). Both anti-αv and anti-α5 were capable of inhibiting the Col15-induced transmigration: anti-αv inhibited the Col15-induced migration by 53% ($P \leq 0.05$) and anti-α5 antibody by 49% ($P \leq 0.05$) (Fig. 4). In combination, anti-α5 and anti-αv inhibited the Col15-induced migration by 58% ($P \leq 0.05$) (Fig. 4). Function-blocking αII$_b$ integrin antibody (CD41a) inhibited the Col15-induced transmigration by 41% ($P \leq 0.05$) and a pharmacological αII$_b$ integrin-blocking drug, tirofiban, by 35% ($P \leq 0.05$) (Fig. 4). Taken together, the data indicate that the carcinoma cell motility promoting function of Col15 is mediated by αv, α5 and αII$_b$ integrins.

Discussion

In the present study, the temporal expression patterns of two interaction partners, collagen XVII and laminin-5, were compared in mucosal wounds and squamous cell carcinomas. In agreement with earlier studies using various wound healing models, laminin-5 γ2 chain was strongly expressed by the leading keratinocytes of the wound, whereas collagen XVII was clearly synthesized by the following keratinocytes in the epithelial outgrowth [20–22]. This is in line with the current

model of hemidesmosome assembly, according to which collagen XVII is incorporated into the molecular complex first formed by laminin-5, α6β4 integrin, and plectin [23]. Our previous study showed a correlation between the enhanced expression of collagen XVII and oral squamous cell carcinoma progression [4]. Similarly, laminin-5 γ2 chain overexpression associates with poor prognosis of colon and tongue carcinomas, and is suggested to function as a predictive marker for microinvasive cervical squamous carcinoma [17,24,25]. Upregulation of both collagen XVII and laminin-5 γ2 chain occurs at the level of gene transcription [4,26]. Here, both laminin-5 and collagen XVII localized to the invading carcinoma cells in the tumor areas protruding into the surrounding tissue. Taken together, collagen XVII and laminin-5 are co-localized in carcinoma progression, but not during wound healing, implicating that collagen XVII expression is differentially regulated during the processes of physiological and malignant migration.

Proteolytic processing of certain ECM proteins may represent a poorly understood mechanism regulating cell migration. There are few examples of ECM macromolecules, such as tenascin-C, laminin-5 and collagen IV, whose function is altered after proteolysis, leading to changes in epithelial cell motility [27–32]. Our present results clearly show that the denatured Col15 domain is capable of functioning as a chemotactic agent by attracting invasive carcinoma cells. Induction of transmigration was more prominent when denatured rather than native, triple-helical Col15 was used. This is in agreement with our previous studies reporting that denatured Col15 functions more effectively in cell adhesion and spreading than its native counterpart [9,16]. In vivo, denaturation of the Col15 domain may occur after the proteolytic shedding of collagen XVII from the epithelial cell surface, resulting in the generation of new receptor or ligand-binding sites. Thus it is possible that the proteolytic remodeling of collagen XVII may lead to alterations in receptor-ligand interactions, affecting signaling pathways and cell behavior.

Expression of Col15-binding integrin subunits α5, αv, and β1 on the surface of HSC-3 carcinoma cells was demonstrated by flow cytometry and immunoprecipitation, and their contribution to the Col15-induced chemotaxis was studied by specific antibody blocking experiments. As expected, β1 integrins proved to be essential for the ability of the cells to migrate through the fibronectin-coated membrane. Blocking of either α5 or αv did not affect the basal level of transmigration, but the combination of α5 and αv integrin antibodies resulted in 58% inhibition, suggesting that α5β1 and αvβ1 integrins may adaptively replace one another when one receptor is nonfunctional [18]. The inhibition by α5 and αv integrin antibodies was greater in the presence of Col15, which implicates that the interaction with α5 and αv integrins plays a role in Col15 chemotaxis. However, blocking of both α5 and αv could not completely prevent the inducing effect of Col15, indicating involvement of additional receptors.

Surprisingly, HaCaT keratinocytes did not show any response to Col15. Analysis of integrin receptor expression reveals some differences between HaCaT keratinocytes and HSC-3 carcinoma cells. HaCaT cells express very low levels of α5β1 integrin [18], whereas an extremely high expression level was detected in a subpopulation of HSC-3 cells. HSC-3 cells expressed large amounts of αvβ1 whereas in HaCaT cells the most prominent αv containing heterodimer was αvβ6 which is known to be responsible of the migration of HaCaT cells on fibronectin [18]. Importantly, carcinoma cells were shown to express the αII_b -integrin subunit, known to be a platelet fibrinogen receptor. In addition to platelets, αII_b is expressed by malignant melanoma cells, but not by keratinocytes [33–35]. Interestingly, integrin-binding sites within Col15 consist of numerous KGD motifs [9] which bind with greater affinity to αII_b than to α5β1 or αv integrins [10]. By blocking the function of αII_b in transmigration assays, it was shown that αII_b integrin is involved in the Col15 chemotaxis.

The results of this study revealed a novel chemotactic function for Col15, a subdomain of collagen XVII, in carcinoma cell migration. In addition to the known collagen XVII binding integrins α5 and αv, Col15 chemotaxis is mediated by integrin αII_b. Our results indicate that, in addition to the previously described melanoma cells [19], some SCC cells also express αII_b and take advantage of this in transmigration. The interaction of αII_b integrin and the Col15 domain of collagen XVII represents a novel molecular interaction in cancer invasion. Importantly, our results indicate that, in addition to antibodies, a drug molecule approved by FDA and used widely all over the world for the treatment of thrombotic diseases, can also inhibit cancer cell transmigration.

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