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Functional relevance of tetraspanin CD9 in vascular smooth muscle cell injury phenotypes: A novel target for the prevention of neointimal hyperplasia

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ABSTRACT

Vascular smooth muscle cell (VSMC) migration and proliferation are critical events in the development of neointima following vascular injury. In this study, we found that CD9 is constitutively expressed in the VSMC of the neointima of injured carotid arteries. The *in vitro* migration and proliferation of human coronary artery smooth muscle (hCASM) cells were reduced by 40% and 63%, respectively, by treatment with a CD9 specific monoclonal antibody mAb7 when compared to control antibody treatment. In a mouse carotid ligation injury model, a single application of a neutralizing anti-mouse CD9 antibody resulted in a 31% (day 14, $n=8$, $p<0.05$), and 32% (day 28, $n=5$, $p<0.01$) reduction in neointima formation. In support of these findings, exogenous expression of human CD9 by CD9-adenoviral transduction led to 43% increases in neointima ($p<0.05$, $n=6$). Upon investigation of the mechanisms underlying CD9 mediated VSMC phenotypic events we found that integrin $\alpha5\beta1$ was a constitutive partner of CD9 and that CD9 significantly augmented PI-3 kinase dependent Akt phosphorylation. Furthermore, enhanced Akt phosphorylation was attenuated by mAb7 binding. Cumulatively, a functional link between CD9, $\alpha5\beta1$, PI3-K/Akt activity and enhanced VSMC migratory and proliferative phenotypes has been demonstrated. These studies suggest that agents that modulate CD9 mediated VSMC phenotypes may emerge as novel strategies for the treatment of abnormal vascular injury response.

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1. Introduction

Vascular injury elicits phenotypic changes in smooth muscle cells leading to neointimal hyperplasia (NH) that predisposes blood vessels to occlusive thrombotic diseases [1]. Vascular smooth muscle cell (VSMC) phenotypic changes and accompanying NH are induced by agents such as growth factors, inflammatory mediators, adhesion molecules, and extracellular matrix (ECM) proteins. VSMC interaction with extracellular matrix via cell surface adhesion molecules controls VSMC migration, proliferation, intracellular signal transduction and remodeling events [2]. In this regard, the role of adhesion receptors, such as the integrins, in regulating VSMC phenotypes is well documented.

The tetraspanin family (TM4SF) of membrane proteins serve as membrane organizers. TM4SF interacts with other membrane proteins such as integrins, Ig superfamily proteins, growth factors and regulate cell phenotypes [3]. CD9 is a 24 kDa TM4SF protein that complexes with β1, β3 integrins [4–6], and with the membrane bound form of growth factors such a pro-HBEGF [7]. Phenotypic effects of CD9 depend upon associated integrins. For example, CD9 functional complexes that include the fibronectin (FN) receptor integrin $\alpha5\beta1$, regulate cell migration on fibronectin [8]. CD9 also modulates phenomenon such as sperm–egg fusion, apoptosis, and proliferation.

CD9 in VSMC associates with β1 integrins [9]. Previous studies showed that blockade of endogenously expressed CD9 using the anti-CD9 mAb, ALMA-1, inhibited VSMC migration [10]. Studies using CD9 knockout (KO) mice, however, did not clarify the role of CD9 in vascular injury response. While vascular injury in CD9 KO mice did result in diminished injury response and decreased

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neointima formation *in vivo* [11], this effect was not statistically significant when compared to wild-type mice. Thus, establishing a definitive role of CD9 in the regulation of vascular injury response and VSMC phenotypes warrants further investigation.

Utilizing previously well-characterized function-blocking reagents for CD9 and CD9 gene transfer, we investigated the relevance of CD9 in VSMC migration, proliferation and in neointima formation. Our findings identified CD9 as a positive regulator of SMC migration and proliferation and revealed putative cellular mechanisms underlying these events. Furthermore, the study established a definitive relationship between CD9, *in vivo* vascular injury response and NH thus identifying CD9 as a novel target for inhibiting adverse VSMC phenotypic response following vascular injury.

2. Materials and methods

2.1. Materials

All chemical reagents, unless otherwise stated, were obtained from Sigma–Aldrich (St. Louis, MO). Vectastain Avidin:Biotinylated enzyme Complex (ABC) kit, 3,3′-diaminobenzidine (DAB) and mouse on mouse (MOM) kits were purchased from Vector Laboratories (Burlingame, CA). BD-Adeno-X virus purification kit was purchased from BD Biosciences (Bedford, MA). DMEM, trypsin-EDTA, and human plasma fibronectin were obtained from Invitrogen (Carlsbad, CA). LY294002 and Protein G Plus/Protein A agarose suspension were from Calbiochem (San Diego, CA). Enhanced chemiluminescence kit and [methyl-^{3}H] thymidine were from GE Health Care (Piscataway, NJ). Protein assay kit was obtained from Bio-Rad (Hercules, CA). Restore™ Western blot stripping buffer was from Pierce (Rockford, IL).

2.2. Antibodies

Rat anti-mouse CD9 mAb (KMC.8), Mouse anti-rat CD9 (RPM.7), rat IgG, and anti-rat α5 (HMα5-1) were from BD Pharmingen (San Diego, CA). Anti-rat-β1 (HMβ1-1), and hamster IgG isotype control were from Biolegend (San Diego, CA). Mouse anti-SMA-α antibody (Clone 1A4) was purchased from Dako (Fort Collins, CO). Fluorescein conjugated anti-mouse IgG, Texas red conjugated anti-rat IgG, biotinylated rabbit anti-rat IgG, biotinylated rabbit anti-mouse IgG, normal rabbit serum, Mouse anti-human CD9 antibody (C-4), rabbit polyclonal anti-CD9 (H-110), and rabbit polyclonal anti-α5 antibody for Western blots were from Santa-Cruz Biotechnology (Santa Cruz, CA). Anti-β1 for Western blot analysis was purchased from BD Transduction Laboratories (San Diego, CA). Anti-human β1 (TS2/16) was a kind gift from Dr. Xin Zhang, UTHSC, Memphis. Anti-actin monoclonal antibody (Clone C4) was purchased from Chemicon (Temecula, CA). Phospho-Akt S473 (9271) and total Akt (9272) antibodies were from Cell Signaling (Beverly, MA). Anti-CD9 mAb mAb7 has been described previously [4].

2.3. Generation of Ad-CD9 virus

Human CD9 adenovirus (Ad-CD9) was generated using an EcoR1 and Xba1 cDNA fragment from the plasmid pRc/CMVp24/CD9 [4] that was subcloned into an E1 deleted adenoviral shuttle vector. The CD9-adenoviral shuttle vector was co-transfected into HEK293 cells with type5 backbone genome plasmid pacAd5 9.2-100, generating Ad-CD9 by *in vivo* recombination. The Ad-LacZ virus was made as previously described [12]. The Ad-CD9 was amplified in HEK 293 cells and isolated with the BD-Adeno-X virus purification kit. RASM cells grown to 80% confluence were infected with either Ad-CD9 or Ad-LacZ control in DMEM medium as described earlier [12].

2.4. VSMC culture and migration

Human coronary artery smooth muscle (hCASM) cells were cultured in smGM2 complete medium as recommended by the manufacturer (Cambrex, Walkersville, MD) and were used between passage 4 and 6 in all experiments. Rat aortic smooth muscle (RASM) cells isolated from Sprague–Dawley rats were cultured in DMEM medium as described earlier [13]. RASM cells at passage 9 were used in the described experiments.

Cell migration was measured via monolayer-wounding assays, as described previously [14]. Briefly, 100% confluent cells were pretreated with serum-free DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 5 mM hydroxyurea for 24 h. Hydroxyurea was used to specifically inhibit cell proliferation during cell migration studies. Cultured monolayers of hCASM were rinsed with DMEM and a ~1 mm wide scratch was made with a sterile pipette tip. Cells were then treated with 2% serum with 1, 10 or 100 μg/ml mAb7 (anti-CD9), 2% serum with 100 μg/ml mIgG (isotype control), serum-free medium (negative control), or 2% serum alone.

RASM cell wound healing assays were performed with cells treated with serum-free DMEM as a negative control, 2% serum as positive control, 2% serum plus 20 μg/ml rabbit polyclonal anti-rat CD9 antibody (H-110), 2% serum plus PI3-kinase inhibitor LY294002 (10 μM), or 2% serum plus LY294002 (10 μM) and 20 μg/ml of anti-CD9 (H-110) antibody ($n = 6$). Data analysis was performed using a computerized image analysis system (Scion Image CMS-800). Cell migration was expressed as the distance migrated in the 24 h time period. Migrated distance (μM) = (distance at scratch − distance after 24 h)/2.

2.5. VSMC proliferation

hCASM cell proliferation was measured using two different methods, cell counting and [^{3}H] thymidine incorporation. hCASM cells were seeded into 24-well plates at 1×10^5 cells/well in complete smGM2 media for 24 h for cell attachment. Complete media was replaced with the serum-free DMEM for 24 h for cell cycle synchronization. Synchronized hCASM cells were then treated in smGM2 media containing 1, 10 or 100 μg/ml mAb7 (anti-CD9) or 100 μg/ml mIgG. For counting, cells were detached by trypsinization 24 h post treatment where cell counts were done using trypan blue exclusion.

For [^{3}H] thymidine incorporation assays, cells were treated as described above, where 3 μCi [methyl-^{3}H] thymidine was added 2 h prior to harvesting. The cells were washed in ice-cold PBS, treated with 10% trichloroacetic acid solution and ethanol: ether (2:1), then air dried at room temperature. Cells were lysed in 500 μl of 0.1% SDS in 0.1N NaOH. 100 μl lysates were added to 3 ml of the EcoLume™ scintillation fluid, and counts were done using a liquid scintillation analyzer (Packard 1900TR).

2.6. Immunoprecipitation and Western blot

Seventy-two hour post transduction, RASM cells were lysed in lysis buffer (1% Brij-98, 10 mM Hepes (pH 7.4), 200 mM NaCl, 5 mM $MgCl_2$, 2 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM phenylmethylsulfonylfluoride (PMSF), 200 μM Na_3VO_4) and protease inhibitor tablets (Roche, Indianapolis, IN). Each immunoprecipitation (IP) reaction (1 mg total protein) was precleared with Protein-G plus Protein-A beads and target proteins immunoprecipitated with 5 μg of specific mAb. Proteins were subsequently captured with the Protein-G plus Protein-A beads and eluted in Laemmli sample buffer and analyzed by Western blot. Western blot analysis for Akt phosphorylation was carried out as previously described [8]. For