Tetraspanin CD9 Is Associated With Very Late–Acting Integrins in Human Vascular Smooth Muscle Cells and Modulates Collagen Matrix Reorganization

Arnaud Scherberich, Sylvie Moog, Gièle Haan-Aarchipoff, David O. Azorsa, François Lanza, Alain Beretz

Abstract—CD9, a member of the tetraspanin family, and very late–acting (VLA) integrins are known to associate and form functional units on the surface of several cell types. We studied the changes in expression of CD9 and β1-integrins (CD29, VLA) in human vascular smooth muscle cells (VSMCs) under in vitro culture conditions mimicking proliferative vascular diseases. We also investigated possible interactions between CD9 and VLA integrins in VSMCs. We found that CD9 is highly expressed in VSMCs and is subject to modulation, depending on the proliferative/contractile state of the cells. In the contractile phenotype, the levels of CD9, CD81, another tetraspanin, and CD29 are ≈50% of those found in the proliferative phenotype. Coimmunoprecipitation experiments showed physical association between CD9 and CD29. CD9 was mainly associated with α5 and α6-integrins (CD49b and c) and also with α5-integrin to a weaker extent. Functionally, the addition of anti-CD9 monoclonal antibodies (MoAbs) doubled the extent of collagen gel contraction mediated by VSMCs, a model for the reorganization of the extracellular collagen matrix occurring in the vessel wall. Anti-CD29 MoAbs inhibited gel contraction, but anti-CD9 MoAbs counteracted this inhibitory effect of anti-CD29 MoAbs. Transfection of human CD9 into Chinese hamster ovary cells more than doubled the extent of Chinese hamster ovary cell–mediated collagen gel contraction (130% stimulation), confirming a role for CD9 in extracellular matrix reorganization. Thus, CD9 seems to be involved in the modulation of VLA integrin–mediated collagen matrix reorganization by VSMCs. These findings suggest that high CD9 expression is associated with a proliferative state of VSMCs. The role of CD9 could be to modulate the function of VLA integrins on the surface of VSMCs. (Arterioscler Thromb Vasc Biol. 1998;18:1691-1697.)

Key Words: CD9 ■ tetraspanins ■ collagen ■ integrins ■ smooth muscle

During atherosclerosis, vascular smooth muscle cells (VSMCs) display various functional changes such as hypertrophy, hyperplasia, and cell migration.1–2 Three main processes are involved in the formation of atherosclerotic lesions: (1) proliferation of VSMCs, as well as macrophages and possibly lymphocytes; (2) formation by VSMCs of the fibrous plaque, a dense, connective-tissue matrix comprising collagen fibers,3 elastic fibrillary proteins, and proteoglycans; and (3) accumulation of lipids enriched in cholesterol in the matrix and the associated cells.4

VSMCs can occur in 2 main distinct states, referred to as a synthetic, or proliferative, phenotype and a contractile phenotype.5 Contractile-state cells have a muscle-like appearance and predominate in normal vessels. However, these cells can revert to the proliferative phenotype, being 1 important early event in atherosclerosis. For example, after mechanical injury of the endothelium, medial VSMCs migrate into the intima and replicate.5 The proliferative, or synthetic, phenotype is fibroblast-like, being characterized by a rapidly dividing state of VSMCs, loss of contractile properties, and production of extracellular matrix components like elastin or collagen.7,8

Fibrous plaques modify arterial biomechanical parameters compared with normal vessels and cause at least instability and sometimes even rupture accompanied by thrombosis and occlusion.9 Collagen matrix reorganization is mediated by α5β1-integrin (very late acting [VLA]2) in many cell types,10,11 including VSMCs.12 Two additional α-subunits (α1 and α5) can form complexes with the β1-subunit (CD29)13 to form VLA1 and VLA3 that also function as collagen receptors.14–16

CD9 antigen, 1 of the leukocyte differentiation antigens, is a 24-kDa surface-membrane glycoprotein17 present on platelets, early B cells, activated T cells, eosinophils, and basophils, as well as nonhematopoietic cells like endothelial...
cells, fibroblasts, or glial cells. CD9 belongs to the tetraspanin, or transmembrane 4, superfamily (TM4SF) that comprises at least 18 cell surface proteins with high sequence homology. Tetraspanins are characterized by the presence of 4 conserved, membrane-spanning, hydrophobic domains and 2 extracellular loops, with both NH2 and COOH termini localized intracellularly (for reviews, see References 18 and 19). Recent reports have shown that CD9 and other members of the tetraspanin family, like CD53, CD63, or CD81 (TAPA-1) associate with VLA integrins in some cell types. The function of CD9 and other tetraspanins is still largely unknown, but CD9 is thought to play a role in cell differentiation and migration. An inverse correlation between metastatic potential and CD9 expression has been documented. Moreover, anti-CD9 monoclonal antibodies (MoAbs) inhibit VSMC migration and augment neutrophil adherence to the endothelium or pre-B cell adhesion to bone marrow fibroblasts. These results led us to investigate the role of CD9 in some functions of VSMCs related to atherosclerosis.

In the current study, we investigated the modulation of CD9 and CD29 expression in human arterial SMCs having different phenotypes. Because many of the biological events leading to atherosclerotic development are VLA-integrin dependent, we looked for an association of CD9 with VLA integrins in VSMCs. By coimmunoprecipitation experiments and collagen gel contraction assays, we showed both a physical and a functional association between this tetraspanin and some VLA proteins. These findings suggest that CD9 could be a marker of proliferative VSMCs and could modulate some of the integrin-dependent functions of these cells.

**Methods**

**Materials**

Aprotinin, antipain, benzamidine, chymostatin, amphotericin B, and streptomycin, penicillin, streptomycin, G418 sulfate, trypsin-EDTA solution, and some VLA proteins. These findings suggest that CD9 and other members of the tetraspanin family, like CD53, CD63, or CD81 (TAPA-1) associate with VLA integrins in some cell types. The function of CD9 and other tetraspanins is still largely unknown, but CD9 is thought to play a role in cell differentiation and migration. An inverse correlation between metastatic potential and CD9 expression has been documented. Moreover, anti-CD9 monoclonal antibodies (MoAbs) inhibit VSMC migration and augment neutrophil adherence to the endothelium or pre-B cell adhesion to bone marrow fibroblasts. These results led us to investigate the role of CD9 in some functions of VSMCs related to atherosclerosis.

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**Flow Cytometric Analysis**

VSMCs in collagen gels (contractile phenotype) were treated for 35 minutes at 37°C with 0.5 mL collagenase (350 U/mL). The cells were rinsed once with PBS containing 1% human albumin and resuspended in FMF medium (RPMI 1640, 5% inactivated goat serum, and 0.2% NaN3). Subconfluent VSMCs in culture flasks were precipitated with protein G–agarose in collagen gel suspensions (2 mL each) were incubated in 6-well plates (Costar) for 1 hour at 37°C to polymerize the collagen, culture medium was then added, and the gels were gently cut away from the sides with a scalpel and lifted off the bottom of the well. At different times thereafter, the diameter of the gels was measured with Starwise image analysis software (Imstar). The same protocol was used for CHO cells with a cellular density of 7.5×10⁵ cells/mL and a 1 mg/mL collagen concentration.

**Immunoprecipitation and Western Blotting**

Detached VSMCs (2×10⁶ per condition) were washed 3× with PBS and resuspended in lysis buffer (1% Triton or 1% CHAPS in PBS, 1 μg/mL aprotinin, 1 μg/mL antipain, 1 μg/mL benzamidine, 1 μg/mL chymostatin, and 0.1 μg/mL acid phosphatase). After a 25-minute incubation on ice, cells were centrifuged at 15 minutes at 10 000 rpm at 4°C. The supernatant was preclarified for 2 hours at 4°C under agitation by incubation in lysis buffer containing 0.01% nonimmune mouse serum and protein G–agarose. After centrifugation for 10 minutes at 3000 rpm, the supernatant was incubated for 1 hour on ice with 30 μg/mL of MoAb and precipitated with protein G–agarose in detergent buffer. After a 1-hour incubation at 4°C under agitation, cells were washed 3× in lysis buffer and twice in 10 mmol/L Tris buffer. The proteins were analyzed by SDS–polyacrylamide gel electrophoresis under nonreduced conditions. They were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) and incubated in 0.1% casein in PBS plus 0.05% Tween (PBS-T). After 3 washes in PBS-T, the membrane was incubated for 1 hour with specific biotinylated MoAbs (10 μg/mL) in PBS-T and 0.2% BSA under constant agitation at room temperature. The membrane was washed 3× with PBS-T and incubated with streptavidin–
horseradish peroxidase. After 3 washes with PBS-T, the bands were revealed by using the ECL chemiluminescence kit (Amersham).

Statistical Analysis of Results
All results are expressed as mean ± SEM. Student’s t test was used, with P < 0.05 considered significant.

Results

In Vitro Models of Contractile and Proliferative Phenotypes

To obtain an in vitro model of the proliferative phenotype, we used cells subcultured on a collagen type I–coated surface. Previous studies have shown a phenotypic reversion to the contractile phenotype with an increase in the volume occupied by myofilaments and in the expression of smooth muscle α-actin mRNA as the cultures become confluent.12 To avoid this phenotypic reversion, we used VSMCs at densities between 10^3 and 4 × 10^3 cells/cm^2. At this density, VSMCs had a marked fibroblast-like appearance and exhibited a doubling time of 24 to 36 hours. To obtain an in vitro model of the contractile phenotype, VSMCs were cultured in 3D collagen gels for 6 days. In this case, VSMCs had a less dendritic and a more elongated morphology compared with proliferating cells and expressed smooth muscle α-actin, whereas the expression of this marker was almost undetectable in proliferating VSMCs (data not shown).

Expression of CD9 and CD29 in Proliferative Versus Contractile Phenotypes

To investigate whether CD9 and/or CD29 could be a potential marker of the phenotypic state of VSMCs, we measured and compared surface expression of both markers by using quantitative flow cytometry. At the cellular density used for the proliferating VSMCs (10^3 to 4 × 10^3 cells/cm^2), the amount of CD9 was twice that of proliferating cultures having higher or lower cell densities (<1000 or 4 × 10^3 to 2 × 10^4 cells/cm^2, data not shown). We showed that CD9 is downregulated when VSMCs approach the contractile phenotype in 3D collagen gels (Figures 1 and 2A). CD81, a tetraspanin closely related to CD9, was similarly regulated. However, the expression of the tetraspanin CD63 was unchanged under the same experimental conditions (Figure 1). The change from the proliferative phenotype to the contractile phenotype was accompanied by a 51% decrease in CD9 surface expression (Figure 2A) and by a 44% downregulation of CD29 surface expression (Figure 2B). The absolute levels of both markers in a given phenotype were very similar, with an ≈1:1 CD9 to CD29 ratio.

Coommunoprecipitation of CD9/CD29 Complexes

To investigate a potential association between CD9 and β1-integrins, we performed coimmunoprecipitation experiments. Under nonstringent conditions with 1% CHAPS, the anti-CD29 MoAb P4C10 coprecipitated a 24-kDa band corresponding to CD9, as revealed by Western blotting with biotinylated anti-CD9 MoAb (Figure 3A). Even under more stringent conditions with 1% Triton X-100, coprecipitation of CD9 with P4C10 was still present but to a much weaker extent. In the reciprocal experiment (Figure 3B), the anti-CD9 MoAb ALMA1 coprecipitated a 110-kDa band corresponding to β1-integrin under nonstringent conditions but not in Triton X-100 lysates (data not shown). The isotype control MoAb MOPC21 coprecipitated neither CD9 (Figure 3A) nor β1-integrin (Figure 3B). The coprecipitation with MoAbs to
α1 to α5-integrins with biotinylated ALMA1 (anti-CD9) showed an association of CD9 with α2 and α3-integrins (Figure 4A) and a weak association with α5. The same experiment with biotinylated C9 (anti-b1 integrin) shows that α2, α3, and α5 are the main α-integrins associated with b1-integrin in VSMCs (Figure 4B).

**Effect of MoAbs Against CD9 and CD29 on Contraction of Hydrated Collagen Gels**

The contraction of hydrated collagen gels was used as a model for the reorganization of the extracellular collagen matrix occurring in the vessel wall. The possible role of CD9 and CD29 in this process was investigated by measuring the effect of anti-CD9 and anti-CD29 MoAbs on the decrease in diameter of hydrated collagen gels with time in culture. The anti-CD9 MoAb ALMA1 stimulated gel contraction by decreasing the delay of onset of contraction compared with untreated cells (Figure 5). Therefore, the extent of contraction was doubled after 72 hours, and there was still 80% stimulation when compared with untreated cells after 96 hours. ALMA3, another anti-CD9 MoAb, produced the same effect (data not shown). Inversely, the anti-CD29 MoAb 4B4 slowed the gel contraction process, with 50% inhibition after 72 hours and 36% after 96 hours. The anti-CD29 MoAb 4C10 produced a similar effect (data not shown). When the 2 MoAbs ALMA1 and 4B4 were added together, there was no significant difference versus untreated cells after 24 hours. A significant stimulation of gel contraction was found after 72 hours, with a 70% increase compared with control. At 96 hours, the effect was superimposable with that obtained with the anti-CD9 MoAb alone.

**Effect of Human CD9 Transfection on Contraction of Hydrated Collagen Gels**

CD9-transfected CHO cells were used in contraction assays to confirm that CD9 is implicated in collagen matrix reorganization. Overexpression of CD9 more than doubled the extent of gel contraction (130% increase after 216 hours) compared with mock-transfected cells (Figure 6). Moreover, the delay of onset of contraction was reduced by 30%, and the speed of contraction was increased by 43% (46.8 versus 32.8 μm/h, P<0.02, n=4).

**Discussion**

The basis for this study originates from results suggesting that CD9 may be implicated in cell migration and adhesion. We thus wanted to investigate the possibility that CD9 plays a role in vascular proliferative diseases. In view of the major role played by VLA integrins in cell-matrix interaction...
The questions asked were the following: (1) Is CD9 expressed in human VSMCs? (2) Is this expression modulated occurring during vascular diseases, we simultaneously measured the expression of CD9 and VLA integrins in VSMCs. The questions asked were the following: (1) Is CD9 expressed in human VSMCs? (2) Is this expression modulated occurring during vascular diseases, we simultaneously measured the expression of CD9 and VLA integrins in VSMCs. The questions asked were the following: (1) Is CD9 expressed in human VSMCs? (2) Is this expression modulated during phenotypic changes? (3) Is there an association of CD9 and β1-integrins? (4) What are the functional implications of the expression of CD9 in VSMCs? We first showed that CD9 is expressed in human VSMCs, confirming previous reports. Quantitative flow cytometry allowed us to estimate a basal expression of 5 × 10^5 CD9 molecules per VSMC. We also showed that the β1-integrin CD29 is expressed in our cells in amounts that are comparable to those of CD9.

Various in vitro models have been proposed to reproduce the contractile phenotype of VSMCs in culture: these include serum starvation and/or the use of heparin, attachment to laminin, or Matrigel, or culture of VSMCs in 3D collagen gels. Collagen gels have been shown to cause differentiation of several cell types. Moreover, fibrillar collagen inhibits arterial SMC proliferation, which is consistent with the low proliferative index of VSMCs in the media of the normal arterial wall. Thus, a 3D culture system may well induce the differentiation of VSMCs toward the contractile phenotype by growth arrest and cell differentiation caused by the surrounding collagen. In our experience, cells placed in 3D collagen gels showed at least a 100-fold increase in smooth muscle α-actin expression compared with proliferative VSMCs (data not shown). We thus compared the expression of CD9 and CD29 in proliferating VSMCs versus cells placed in 3D collagen gels.

We wished to investigate whether the expression of CD9 and CD29 was modified during these phenotypic changes. The expression of CD9 and CD29 in the proliferative phenotype is twice that in the contractile phenotype (Figure 2). However, this phenotypic modulation was not a nonspecific consequence of the experimental procedures used to obtain the different phenotypes, since the expression of another tetraspanin, CD63, was not modified from 1 phenotype to the other (Figure 1). We can also notice that CD81, a tetraspanin known to associate with CD9 and CD29 in many cell types, is similarly upregulated when approaching the proliferative phenotype. The anti-β1-MoAb used does not discriminate between the various VLA subtypes, and preliminary results show that variations in the expression of individual VLA integrins seem to occur. We also found that the CD9 to CD29 ratio is close to 1 for both phenotypes. Our experiments show an expression of ≈5 × 10^5 molecules of CD9 per VSMC in proliferating cells. This high level of CD9 expression and its phenotypic modulation, which parallels that of CD29, suggest that CD9 and VLA integrins could play a critical role in some of the cellular processes involved in the phenotypic change of VSMCs. Moreover, in other cell types, CD9 and other tetraspanins as well as CD29 have been implicated in cellular processes known to occur in the development of atherosclerotic lesions, including cell adhesion, motility, and proliferation. This finding supports a potential role for CD9 and CD29 in atherosclerotic and restenotic processes.

An association between CD9 and VLA integrins in this cell type is supported by coprecipitation of CD9 and β1-integrin in VSMCs (Figure 3). CD9 and other members of the tetraspanin family, like CD53, CD63, CD81, and CD82, have already been shown to associate with β1-integrins in several cell types but never before in vascular cells. CD9 has been reported to associate with VLA3, VLA4, and VLA6 but not with VLA1 integrins. Interaction with VLA5 seems so far restricted to pre-B cells and interaction with VLA2 to human epidermal keratinocytes. In coprecipitation experiments, we showed an association between CD9 and VLA2 and VLA3 and a weak association of CD9 with VLA5 (Figure 4). Previous studies by Lee et al showed that VSMCs express abundant levels of VLA1, VLA2, VLA3, and VLA5, whereas the cell surface...
expression of VLA4 is low and VLA6 is almost undetectable. In our cells, the pattern of VLA surface expression was VLA2>VLA3>VLA5>VLA1>VLA4>VLA6, as tested by quantitative flow cytometry (data not shown). It is well possible in this case that the VSMCs cultured on type I collagen could induce the overexpression of α2 and α5-integrins, which are known to be collagen receptors. Additional studies will be necessary to determine the importance of the extracellular matrix on the association of CD9 with distinct VLAs in VSMCs.

Collagen matrix reorganization is implicated in the stiffening of atherosclerotic tissue. Contraction assays of hydrated collagen gels suggest that the CD9/CD29 association could result in a functional coupling. We showed that anti-CD29 function-blocking antibodies inhibit the contraction of the collagen gel by VSMCs, probably by inhibiting the VLA2 integrin in accord with the results of Lee et al. In contrast, the 2 anti-CD9 MoAbs tested led to an earlier onset of gel contraction. In view of the opposing effect of CD9 and VLA integrin MoAbs (Figure 5), we tested the effects of both antibodies together. The anti-CD9 MoAb counteracted the effect of the anti-β1-integrin MoAb on gel contraction. Moreover, transfection of CD9 into CHO cells led to increased speed and shorter delay of onset of collagen gel contraction mediated by these cells (Figure 6). These results implicate CD9 in the reorganization of the extracellular matrix and suggest that the association to VLA integrins may serve to either amplify or regulate the ability of VLA to reorganize collagen lattices. However, the cellular effect of anti-CD9 MoAbs is largely unknown. In the absence of any evidence for a direct interaction of CD9 with collagen, we can speculate that CD9 is a regulating subunit of VLA integrins implicated in collagen reorganization. This regulation could occur at 2 levels. CD9 could interfere with integrins in their binding to extracellular ligands and/or in the transduction of integrin-dependent intracellular signals. In the latter hypothesis, the anti-CD9 MoAb could stimulate intracellular events through its binding to CD9, leading to a modulation of β1-integrin–dependent contraction. In this respect, CD9 could act as a “molecular facilitator” of integrin signaling complexes, as proposed by Maecker et al.

The pathways of cell activation after CD9 stimulation are largely unknown, except in the case of platelet activation. In platelets, anti-CD9 antibodies lead to the stimulation of the FcγR by the Fc region of the antibody, whereas the variable part is bound on CD9. This induces an increase in intracellular calcium, which is therefore only partially dependent on CD9. Using fura 2–loaded VSMCs, we found no increase in intracellular Ca2+ after stimulation with anti-CD9 MoAbs. We also showed by flow cytometry the absence of FcγRs in VSMCs (data not shown). Therefore, the effect of anti-CD9 MoAbs on gel contraction is not due to an Fc-dependent increase in Ca2+. However, this does not exclude the possibility that binding of the MoAb to CD9 could have other intracellular effects. Recently, a direct effect of an anti-CD9 F(ab)2 fragment has been reported involving the activation of the nonreceptor tyrosine kinase p72SYK. Activation of a tyrosine kinase could thus be implicated in the stimulatory effect of the anti-CD9 antibody in collagen gel contraction. VLA integrins, when activated by fibrillar proteins such as collagen or fibronectin, phosphorylate tyrosine kinases such as p125SYK. CD9, through its ability to physically associate with VLA integrins, could interfere with p125SYK tyrosine phosphorylation. The hyaluronan receptor RHAMM is another example of a nonintegrin membrane receptor that is able to mimic α2β1 stimulation by restoring the phosphorylated state of p125SYK or other tyrosine kinases.

In conclusion, we propose that CD9 could be a potential marker of the atherosclerotic state of blood vessels. Indeed, our study shows an increase in CD9 surface expression in cultured VSMCs when they switch from the contractile to the proliferative phenotype. There are precedents linking overexpression of tetraspanin molecules in the course of the atherosclerotic process. Indeed, Xu et al have shown that CD9 is upregulated during monocyte/macrophage differentiation. The mRNA for CD63, another member of the tetraspanin family, is increased in atherosclerotic lesions or in aged vessels. The hypothesis that CD9 is a potential marker of the atherosclerotic state of vessels will have to be confirmed by comparing CD9 expression in normal and atherosclerotic tissues. CD9, by its association with VLA integrins, could play an important role in atherosclerosis by modulating the collagen matrix reorganization, a process involved in the thickening of the blood vessel. Indeed, we showed in this study that MoAbs directed against CD9 can counteract the inhibition of gel contraction induced by anti-CD29 MoAbs, which is considered a good model of collagen matrix reorganization in atherosclerosis. Moreover, independent studies have shown that anti-CD9 antibodies can counteract VSMC migration, another key cellular event occurring in atherosclerotic lesions, probably by the inhibition of the migration signal induced by VLA integrins. The stimulation of collagen matrix reorganization and the inhibition of VSMC migration by MoAbs against CD9 speak for CD9 as a potential target in the treatment of proliferative diseases like atherosclerosis or restenosis after coronary angioplasty.

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References


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