Regulation of α7-integrin expression in vascular smooth muscle by injury-induced atherosclerosis

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Chao, Jun-Tzu, Gerald A. Meineinger, Jan L. Patterson, Sarah A. L. Jones, Charles R. Partridge, Jessey D. Neiger, E. Spencer Williams, Stephen J. Kaufman, Kenneth S. Ramos, and Emily Wilson. Regulation of α7-integrin expression in vascular smooth muscle by injury-induced atherosclerosis. Am J Physiol Heart Circ Physiol 287: H381–H389, 2004. First published February 26, 2004; 10.1152/ajpheart.00939.2003.—Injury of vascular smooth muscle cells (VSMCs) by allylamine (AAM) leads to phenotypic changes associated with atherogenic progression including increased proliferation, migration, and alterations in cell adhesion. In the present study, the relationship between AAM-induced vascular injury and expression, migration, and alterations in cell adhesion. In the present study, the relationship between AAM-induced vascular injury and expression of the α7-integrin subunit was investigated. The α7-mRNA and protein expression were examined using real-time RT-PCR, fluorescence-activated cell sorting analysis (FACS), immunohistochemistry, and immunoblotting. In cultured VSMCs from aortas of AAM-treated rats (70 mg/kg for 20 days), α7-mRNA levels were increased more than twofold compared with control cells. No change was seen in β1-integrin expression. FACS analysis revealed increased cell surface expression of α7-protein (25 ± 9%; *P < 0.05). AAM treatment of naïve VSMCs enhanced α7-mRNA expression (2.4 ± 0.7-fold, mean ± SE; *P < 0.05). The increased α7-mRNA expression was attenuated by the amine oxidase inhibitor semicarbazide and the antioxidant pyrrolidine dithiocarbamate, which confirms a role for oxidative stress in modulating α7-expression. In vivo α7-mRNA and protein expression were enhanced in the aortas of AAM-treated rats. In addition, increased α7-integrin expression facilitated AAM VSMC adhesion to laminin more efficiently compared with control (51 ± 2%; *P < 0.05). Chemical injury induced by AAM significantly enhances α7-integrin expression in VSMCs. These findings implicate for the first time the expression of α7-integrin during the response of VSMCs to vascular injury.

α7-integrin; aorta; cell adhesion; laminin; vascular injury

INTEGRINS PLAY ESSENTIAL ROLES in the regulation of a variety of cell functions including cell adhesion, migration, proliferation, and cell survival (30). Among these functions, integrins are also involved in organizing the linkage between the extracellular matrix and the cytoskeleton and in engaging signal transduction pathways (41–43). In addition, altered integrin expression has been linked to vascular dysfunction (31). Despite growing evidence associating altered integrin expression with vascular dysfunction, the extent to which alterations in integrin expression correlate with various types of vascular injury is not well understood.

Previous reports have demonstrated that repeated cycles of injury by allylamine (AAM), a synthetic amine metabolized by semicarbazide-sensitive amine oxidase to acrolein and H2O2, cause atherosclerosis (27) and induce proliferative vascular smooth muscle cell (VSMC) phenotypes that are maintained over serial passages in culture (11). Proliferative AAM cells are characterized by increased secretion of osteopontin, a component of atherosclerotic lesions (12), and are accompanied by increased protein expression of β3-integrin, an osteopontin integrin-receptor subunit (29). Additionally, AAM-injured VSMCs display reduced expression of α1- and α5-integrin subunits and enhanced downstream integrin-coupled signaling events associated with increased proliferation. These signaling-linked events include increased focal adhesion kinase activity and increased activator protein-1 and nuclear factor-κB binding activity (47). The proliferative phenotype afforded by AAM cells is abolished when the cells are grown on type I collagen, which suggests an important role for integrin signaling in growth modulation of injured VSMCs. Taken together, these findings are consistent with the hypothesis that the AAM-induced proliferative advantage of VSMCs is modulated through altered integrin expression and associated integrin signaling. Therefore, the purpose of this study was to investigate the expression of specific integrins in AAM-injured VSMCs. For example, α3- integrins were previously implicated in vascular pathology linked to atherogenesis (5, 16), but little is known about the role of other integrins in atherogenic VSMCs. Of particular interest is the α7-integrin; this integrin was identified by microarray analysis to be highly regulated by AAM in VSMCs (Partridge et al., unpublished observations).

The laminin-binding αβ1-integrin is expressed predominantly in skeletal and cardiac muscles (44). The pathophysiological significance of its expression is demonstrated by increased αβ1-integrin expression in patients with Duchenne muscular dystrophy (15). Enhanced expression of αβ1-integrin is also detected in skeletal muscle of mice with a defective dystrophin gene (15). In addition, enriched αβ1-integrin immunostaining intensity is found in regenerating myofibers after injury by transection (22). These results provide evidence that expression of α7-integrin may be involved in tissue regeneration after injury. Additionally, a recent study using a pressure-overload...
model of cardiac hypertrophy demonstrated increased α7-protein expression in overloaded left ventricles (3). This implicates increased α7-integrin levels in cardiovascular dysfunction. Based on these observations, we investigated α7-integrin expression levels in AAM-induced vascular injury. In this study, we used three model systems as follows: 1) established cultures of VSMCs isolated from rats administered AAM for 20 days in vivo, 2) naive VSMCs treated acutely with AAM in vitro, and 3) freshly isolated arterial sections from AAM-injured rats (in vivo) to provide evidence for the first time that the expression of α7-integrin is regulated in response to vascular injury.

**MATERIALS AND METHODS**

The experimental protocol was in accord with guidelines published by National Institutes of Health and was approved by the Texas A&M University Laboratory Animal Care Committee. All rats used in the study were treated humanely and in accordance with those guidelines.

**Cell culture.** VSMCs were isolated by enzymatic digestion of aortas from Sprague-Dawley rats gavaged with AAM hydrochloride (Sigma; St. Louis, MO) or tap water as previously described (11). Cells were grown in medium 199 (M199; Invitrogen; Carlsbad, CA) supplemented with 10% FBS (Hyclone; Logan, UT), 2 mmol/l glutamine, 100 units penicillin, and 100 μg streptomycin (Invitrogen) in 5% CO2-95% air at 37°C. These cells were designated as established AAM or control VSMC cultures, respectively, and used as matched pairs between passages 19 and 24. Naïve VSMCs isolated independently from normal Sprague-Dawley rats were cultured in the same manner. These cells were designated as naïve VSMCs and were used between passages 9 and 15 to verify that passage number is not a primary factor in α7-integrin expression. Naïve VSMCs were quiesced by serum starvation (0.5% FBS) in M199) for 48 h before AAM treatment (0, 10, 50, and 100 nmol/l for 4 h and 50 nmol/l for 1.5 or 4 h). Thus the cells were predominantly in G0 phase of the cell cycle (21) and were confluent at the time of treatment. At the end of treatment, cultures were either processed for RNA isolation or incubated for an additional 18 h in 10% FBS in M199. RNA was isolated after the designated incubation period. Pyridoline dihydrocarbamate (PDTC), semicarbazide, and all other reagents were purchased from Sigma.

**RNA quantification.** Cellular RNA was isolated using RNAqueous kit (Ambion; Austin, TX) according to the manufacturer’s protocol. Aortas were removed from control and AAM-treated rats at the end of the dosing period, snap-frozen in liquid nitrogen, and stored in −80°C until being processed for RNA isolation. Frozen aortas were suspended in 1 ml TRIzol (Invitrogen) before a brief homogenization (at 0°C) and total RNA extraction. RNase-free DNase (Ambion) was used to remove genomic DNA contamination. Reverse transcription was performed with either total cellular (500 ng) or aortic (200 ng) RNA using ThermoScript reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. As a negative control, equal amounts of total RNA were processed as described above without reverse transcriptase. Transcribed cDNA (3 μl) was amplified by standard real-time PCR performed with an ABI 7700 sequence-detection system (Applied Biosystems; Foster City, CA). PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Forward and reverse primers and probes were designed using Primer Express 1.5a software (Applied Biosystems; sequences are listed in Table 1). Primers were purchased from Sigma-Genosys (Woodlands, TX), and probes were purchased from Applied Biosystems. The ΔΔCt method was used to quantify integrin mRNA expression. Amplification efficiency of α7-, α5-, α6-, α7-, β1-, and β3-integrin was verified to be approximately equal to amplification efficiency of internal reference 18S RNA over a range of 0.9–90 ng of RNA per reaction. Results are presented relative to the expression level of 18S RNA.

**Fluorescence-activated cell sorting analysis.** VSMC surface protein expression of α7- and β1-integrins was assayed by flow cytometry using a fluorescence-activated cell sorting analysis (FACS) Calibur apparatus (Becton Dickinson; Franklin Lakes, NJ) with CellQuest software (Becton Dickinson). Random cycling control and AAM VSMCs were washed and detached with Versene (1:5,000 dilution in PBS; 0.2 g/l EDTA-4Na; Invitrogen) at 37°C for 5 min. Detached cells were incubated with 5% BSA (USBiochemical; Piscataway, NJ) to block nonspecific binding. Cells were then incubated with either fluorescein-5-isothiocyanate (FITC)-conjugated hamster anti-rat β1-monoclonal antibody (BD Pharmingen; San Diego, CA) or mouse anti-rat α7-integrin antibody (H36; 1:500 dilution; provided by S. J. Kaufman) on ice for 1 h (44). For detection of α7-integrin, VSMCs were incubated with FITC-conjugated goat-anti-mouse IgG (BD Pharmingen) for 30 min on ice. Incubations with FITC-conjugated hamster IgM isotype control (BD Pharmingen) or FITC-conjugated mouse IgG isotype (BD Pharmingen) in the absence of primary antibody were used as negative controls. For determination of the fluorescence intensity, 10,000 cells/sample were counted. Values of <2% of total positive fluorescence were set as negative expression.

**Immunofluorescence imaging.** Aortas isolated from control and AAM-injured rats were prepared according to the ELF-97 Immunochemistry Protocol (MP 06600; Molecular Probes; Eugene, OR). Expression of α7-integrin in aortas was determined by immunofluorescence microscopy as described by Kingsley (26), except α7-expression was detected using H36 anti-α7 antibody (1:500 dilution in washing buffer: 30 mmol/l Tris-HCl and 150 mmol/l NaCl, pH 7.5). All reactions were performed in a humidified chamber at room temperature. Stained tissues were visualized using a Bio-Rad ( Hercules, CA) RTS 200MP confocal microscope equipped with 488 nm diaminodimino-2-phenylindole dihydrochloride (DAPI) long-pass (LP) and FITC filter sets. ELF-substrate excitation and emission wavelengths were 360 and 535 ± 18 nm, respectively. A UV band-pass (BP) excitation filter (340–380 nm) and an LP suppression filter (425 nm) were used for the collection of DAPI images.

**Immunoblotting.** Aortas isolated from control and AAM-treated rats were pulverized under liquid nitrogen and solubilized in 100–200 μl of lysis buffer that contained 20 mmol/l Tris-HCl (pH 7.4), 50 mmol/l NaCl, 50 mmol/l NaF, 50 mmol/l EDTA, 20 mmol/l sodium pyrophosphate, 1% Triton X-100, 0.1% sodium deoxycholate, and 1X protease inhibitor cocktail that contained 500 μmol/l 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 150 mmol/l aprotonin, 1 μmol/l E-64, 0.5 mmol/l EDTA, disodium salt, and 1 μmol/l leupeptidase.
Allylamine Increases \( \alpha_\gamma \)-Integrin Expression in VSMCs

**RESULTS**

**Integrin expression in AAM-injured VSMCs.** Previous observations from this laboratory demonstrated altered protein expression of integrins in cultured VSMCs from AAM-treated rats (47). In the present study, we screened cultured VSMCs derived from control and AAM-treated rats for alterations in mRNA expression of \( \alpha_1 \), \( \alpha_4 \), \( \alpha_7 \), \( \alpha_\gamma \), \( \beta_1 \), and \( \beta_3 \)-integrins. As shown in Fig. 1, \( \alpha_\gamma \)- and \( \alpha_\gamma \)-mRNA expression levels were increased by 2.4- and 6.7-fold, respectively (*\( P < 0.05 \)) in randomly cycling AAM VSMCs compared with control cells. No significant difference was observed in the expression of \( \beta_1 \), which is the major integrin binding partner of \( \alpha_\gamma \), between AAM VSMCs and control cells (Fig. 1). Also, no significant change was observed in \( \alpha_1 \), \( \alpha_4 \), and \( \beta_3 \)-mRNA levels.

We next investigated the effects of AAM on cell surface protein expression of \( \alpha_\gamma \) and \( \beta_3 \)-integrins by FACS analysis. As shown in Fig. 2, A and B, cell surface protein expression of \( \alpha_\gamma \) was increased (25 \( \pm \) 9%; *\( P < 0.05 \)) in randomly cycling AAM cells compared with control cells, whereas expression of \( \beta_1 \) was not altered relative to control (Fig. 2, C and D).

**Regulation of \( \alpha_\gamma \)-expression by AAM.** To determine whether acute treatment with AAM directly regulated \( \alpha_\gamma \)-integrin levels, naive VSMCs were treated with AAM for various times and doses, and \( \alpha_\gamma \)-mRNA levels were determined. Figure 3A demonstrates a dose-dependent increase in \( \alpha_\gamma \)-mRNA levels. No change in \( \alpha_\gamma \)-mRNA levels was observed at 10 nmol/l of AAM, but a significant increase in \( \alpha_\gamma \)-integrin mRNA level was observed with 50 nmol/l of AAM (2 \( \pm \) 0.35; *\( P < 0.05 \) vs. naive). Increased \( \alpha_\gamma \)-integrin mRNA levels were also observed with 100 nmol/l AAM; however, substantial cell loss was observed at the end of the 4-h treatment. Therefore, 50 nmol/l was used in subsequent studies.

As shown in Fig. 3B, \( \alpha_\gamma \)-mRNA levels increased slightly at 1.5 h, whereas a significant increase was observed (2.5-fold; *\( P < 0.05 \)) after 4 h of AAM treatment. No difference was observed at earlier time points (data not shown). Notably, upon removal of AAM and return of cells to normal growth medium for 18 h, \( \alpha_\gamma \)-mRNA levels returned to the basal level. These results indicate that sustained stimulation by AAM is required for increased \( \alpha_\gamma \)-mRNA levels.

To investigate the potential mechanism of AAM-induced \( \alpha_\gamma \)-expression, control and AAM-injured VSMCs isolated from rat aortas were incubated with PDTC (100 nmol/l for 4 h), which is an antioxidant that inhibits activation of the transcription factor nuclear factor-\( \kappa \)B (NF-\( \kappa \)B; Refs. 8, 20). As shown in Fig. 4A, \( \alpha_\gamma \)-mRNA levels were inhibited by 83 \( \pm \) 16% in AAM cells treated with PDTC (**\( P < 0.05 \)). The reduction of \( \alpha_\gamma \)-levels was not due to a cytotoxic effect of PDTC, because no cell loss was observed at the end of treatment as determined by Trypan blue exclusion (data not shown). Control cells showed a slight increase in \( \alpha_\gamma \)-levels, which is consistent with a potential prooxidant effect of PDTC (24, 25). These studies suggest a role for oxidative stress in regulating \( \alpha_\gamma \)-expression in AAM-injured VSMCs.

To determine whether metabolism of AAM was essential for AAM induction of \( \alpha_\gamma \)-integrin in naive VSMCs, we investigated the effects of semicarbazide, a selective inhibitor of the amine oxidase, on \( \alpha_\gamma \)-integrin levels. Figure 4B shows that \( \alpha_\gamma \)-mRNA levels were increased significantly by in vitro treatment with AAM, and this increase was attenuated by 21 \( \pm \) 8% in the presence of 100 nmol/l semicarbazide (**\( P < 0.05 \) vs. AAM). The change in expression level was not due to cytotoxicity as no cell loss was observed at the end of semicarbazide incubation (determined by Trypan blue exclusion; data not shown).
To determine whether AAM regulated \( \alpha_7 \)-mRNA expression at the transcriptional level, naive VSMCs were treated with AAM in the presence of 2 \( \mu \)g/ml of either actinomycin D, an inhibitor of RNA synthesis, or cycloheximide, an inhibitor of protein synthesis, for 4 h. AAM-stimulated \( \alpha_7 \)-mRNA expression in VSMCs was attenuated by 31% (*\( P < 0.05 \)) in the presence of actinomycin D, whereas no significant difference was observed after cycloheximide treatment. These data suggest that the regulation of \( \alpha_7 \)-expression by AAM is mediated partially at the transcriptional level and that de novo protein synthesis is not necessary.

Integrin expression in aortas from AAM-injured rats. To determine whether changes in \( \alpha_7 \)-integrin level observed in cultured VSMCs were relevant to alterations seen in vivo, the effects of AAM on \( \alpha_7 \)-integrin levels were studied in isolated aortas. Total RNA was isolated from aortas of control and AAM (70 mg/kg of body wt)-treated rats. As shown in Fig. 5, a significant increase of \( \alpha_7 \)-mRNA levels (1.8 ± 0.3-fold; *\( P < 0.05 \)) was observed in AAM-treated rats compared with controls; no change in \( \beta_1 \)-integrin level was observed.

We next examined whether the protein level of \( \alpha_7 \) was altered in AAM-injured aortas by immunofluorescence histochernistry. Cell nuclei were stained with DAPI (shown in white). Elastin is shown by autofluorescence (gray). Punctate staining of \( \alpha_7 \) was selectively found in aortas from AAM-treated animals (Fig. 6A). Additionally, discontinuous and fragmented changes in elastic lamina were only observed in AAM-treated aortas, which indicates injury to the aortic wall (Fig. 6A).

To verify the results of the immunofluorescence imaging, immunoblot analysis was also performed on protein lysates from aortas isolated from control and AAM-treated rats. As
shown in Fig. 7, α7-integrin protein was consistently present at higher levels in aortas from AAM-treated rats compared with the control aortas (3.5 ± 0.54-fold; *P < 0.05) as determined by densitometry of the resulting bands. This result further

confirms our findings that α7-integrin levels were increased significantly in AAM-injured aortas.

To determine whether the increased level of α7-integrin in AAM VSMCs corresponded to increased adhesion to laminin, cell adhesion assays were performed. As shown in Fig. 8, adhesion to laminin is more efficient in AAM VSMCs than in control cells (51 ± 2%; *P < 0.05). This adhesion was inhibited by anti-α7 antibody (34 ± 2%; *P < 0.05). No difference in adhesion was observed when cells were incubated with mouse IgG (data not shown); this indicates that the inhibitory effect of anti-α7 antibody was specific. These data suggest that enhanced α7-integrin expression in AAM VSMCs may facilitate increased adhesion to laminin.

DISCUSSION

AAM is an atherogenic amine characterized as a model of chemically induced atherosclerosis (6, 27). Oxidative injury to the vessel wall by AAM is mediated by metabolic conversion of AAM to acrolein and hydrogen peroxide by a vascular-specific amine oxidase. This oxidative deamination is inhibited by semicarbazide, a selective inhibitor of the amine oxidase (7). Recent studies from this laboratory suggest that aberrant integrin expression is involved in the induction of proliferative VSMC phenotypes in response to AAM and that the integrin-matrix interaction modulates proliferative VSMC phenotype (47). To further investigate the effects of AAM on integrin expression, we screened control and AAM cells for mRNA expression of α1- and β1-integrin mRNA in aortas from AAM-injured aortas. The mRNA levels of α7- and αv-integrins were increased significantly in AAM-injured VSMCs. Expression levels of all other integrin subunits studied were not changed.

A significant increase of α7-mRNA levels in AAM-injured VSMCs is demonstrated (see Fig. 1). These data are compatible with previous findings from others implicating increased α7-integrin levels in vascular dysfunction (16, 38), and αvβ3-integrin was shown previously to be important in the AAM phenotype. For example, the proliferative phenotype of AAM was abolished in the presence of anti-αv antibody and RGD-containing peptides (Arg-Gly-Asp) that bind to αvβ3-integrin (32, 47). Although αv-mRNA levels were increased by sixfold in AAM-injured VSMCs in the present study, we
previously reported no significant increase in the cell surface protein level of \(\alpha_v\)-integrin (47). However, there was an increase in the \(\beta_3\)-integrin (47) that is consistent with an increase in \(\alpha_v\beta_3\)-cell surface levels. As the significance of the elevated expression of \(\alpha_v\beta_3\)-integrin in the AAM phenotype has been documented (32, 47), we focused our subsequent studies on \(\alpha_v\beta_3\)-integrin.

The extent to which there are common regulatory pathways modulating both \(\alpha_v\)- and \(\alpha_v\beta_3\)-integrin levels was not investigated in this study. Nonetheless, from murine promoter analysis, binding sites for transcription factors Sp-1, Ap-2, and Ets reside in both \(\alpha_v\)- and \(\alpha_v\beta_3\)-promoters (23, 51), and these binding sites have been shown to be redox sensitive (17, 34, 39), which suggests the possibility of common regulatory pathways involved in increased \(\alpha_v\)- and \(\alpha_v\beta_3\)-integrin levels.

The modulation of \(\alpha_v\beta_3\)-integrin in vascular injury has not been reported previously. Therefore, the increased expression of \(\alpha_v\beta_3\)-integrin in cells from AAM-injured aortas was of particular interest. The induction of \(\alpha_v\beta_3\)-integrin in VSMCs by AAM-induced vascular injury is supported by the following evidence: 1) increased \(\alpha_v\beta_3\)-mRNA and cell surface protein levels were observed in VSMCs isolated from AAM-treated rats (see Figs. 1 and 2, A and B); 2) a dose- and time-dependent increase in \(\alpha_v\beta_3\)-mRNA levels occurs in naive VSMCs treated with AAM in vitro (see Fig. 3); 3) elevated \(\alpha_v\beta_3\)-mRNA levels were attenuated by PDTC in VSMCs cultured from AAM-injured aortas and by semicarbazide in naive VSMCs treated with AAM, respectively (see Fig. 4, A and B); 4) the presence of \(\alpha_v\beta_3\)-protein was increased in the aortas of rats subjected to AAM treatment (see Figs. 6A and 7).

Previous studies from our laboratory and others have implicated oxidative stress as a mechanism by which AAM exerts its vascular toxicity (1, 2, 35, 36). A role for oxidative stress in AAM-induced \(\alpha_v\beta_3\)-expression is based on the following evidence: 1) In VSMCs, AAM is metabolized by a vascular-specific amine oxidase that produces hydrogen peroxide and acrolein, a highly reactive monoaldehyde that readily induces oxidative injury (33). 2) In the presence of PDTC, which is an antioxidant and an inhibitor of NF-\(\kappa\)B activation, increased...
\(\alpha_7\)-integrin expression in VSMCs cultured from AAM-treated rats in vivo is attenuated significantly. Also, increased NF-\(\kappa B\) activation is present in AAM VSMCs, and the proliferative advantage of AAM VSMCs is inhibited by PDTC (47). As the regulatory mechanism of \(\alpha_7\)-integrin expression in VSMCs is not fully understood, the possibility of NF-\(\kappa B\) involvement in the direct or indirect signaling cascade that regulates \(\alpha_7\)-integrin level cannot be excluded.

3) Using the specific vascular amine oxidase inhibitor semicarbazide, acute stimulation of \(\alpha_7\)-integrin expression by AAM in naive VSMCs is inhibited significantly. These data support the role for oxidative stress in mediating AAM-induced \(\alpha_7\)-integrin expression.

Regulation of the expression of other integrins by alteration in cellular redox status was previously reported. For example, reduced expression of \(\alpha_1\), \(\alpha_5\), \(\alpha_\gamma\), and \(\beta_\gamma\)-integrins in a human erythroleukemia cell line in the presence of \(\alpha\)-tocopherol (a known cellular antioxidant) was reported by Breyer et al. (9). Likewise, PDTC inhibits CD11a lymphocyte function-associated antigen (LFA)-1 \(\alpha\)-subunit expression in cells of myeloid lineage (14). Thus, precedent lends credence for oxidant-mediated regulation of integrin expression.

The observation that inhibition of \(\alpha_7\)-integrin expression by PDTC is considerably more effective than semicarbazide may arise from the difference in the experimental models used. The effects of PDTC were determined using VSMCs isolated from AAM-treated rats (20 days), whereas the effects of semicarbazide were determined in naive VSMCs treated acutely with AAM. Also, a significant increase of amine oxidase activity with increased cell passage number was reported previously (19, 46). This observation may account for the partial inhibition at the concentration of semicarbazide used (100 \(\mu\)mol/l).

The effects of semicarbazide on \(\alpha_7\)-integrin expression in established AAM VSMCs were not determined, because the AAM treatment was performed in vivo and VSMCs were isolated immediately after treatment.

To determine whether there was a direct effect of AAM on \(\alpha_7\)-integrin level, naive VSMCs were treated with AAM in vitro for short periods of time. The \(\alpha_7\)-mRNA was increased slightly after 1.5 h of treatment with AAM (50 \(\mu\)mol/l) and was enhanced significantly after 4 h; this was not due to increasing cell density, because naive cells also reached confluence at the end of 48 h of quiescence. The \(\alpha_7\)-mRNA levels after longer incubation periods were not determined; however, after an 18-h recovery period that followed acute AAM stimulation, \(\alpha_7\)-mRNA levels returned to the basal level. These data show that AAM-induced \(\alpha_7\)-expression in naive VSMCs is reversible such that when the source of injury (i.e., AAM) is removed, \(\alpha_7\)-mRNA expression returns to the basal level.

The transient expression observed after acute in vitro treatment contrasts with that seen in long-term passages of AAM-injured VSMCs derived from rats treated chronically with AAM (see Fig. 1) in which increased \(\alpha_7\)-integrin expression is maintained at higher levels compared with control cells. Our previous studies demonstrated that proliferative advantage and the characteristic phenotype of AAM cells compared with control cells are persistent with increasing passages (21, 32, 37, 47). The preservation of these characteristics with serial passages is attributed to epigenetic changes in the cells due to chronic in vivo treatment leading to an altered phenotype that is characterized by an altered redox status. In contrast, the acute treatment in vitro with AAM does not cause permanent changes in naive VSMCs. The difference in \(\alpha_7\)-integrin levels may also be attributed to differences in the bioavailability, metabolism, and longevity of the amine in vitro vs. in vivo.

Overall, our data are comparable with the findings of Kaariainen et al. (22) that \(\alpha_7\)-integrin expression is enhanced in myofibers injured by transection and that expression returns toward a normal level after reestablishment of firm adhesion to the extracellular matrix. Taken together, these observations suggest a potential function for \(\alpha_7\)-integrin expression during tissue injury in the vasculature and the remodeling process.

Present knowledge regarding the regulatory mechanisms of \(\alpha_7\)-integrin expression in VSMCs is limited. The gene promoter of murine \(\alpha_7\)-integrin in skeletal myoblasts has been characterized (51). Five putative Sp-1 binding sites, eight consensus E-boxes providing the binding sites for basic helix-loop-helix (bHLH), two Ap-1 binding sites, one Ap-2 binding site, and one Ets binding site have been identified within the 5'-flanking region. Although it is not known whether these elements and their transcription factors play a role in AAM-induced \(\alpha_7\)-expression in VSMCs, Ap-1, Ap-2, and Ets are activated by reactive oxygen species (17, 18, 39). Sp-1 and bHLH proteins have also been implicated in redox signaling in VSMCs (34). Thus, redox-sensitive transcription factors may participate in the regulation of \(\alpha_7\)-integrin expression in AAM-injured VSMCs.

The increased expression of \(\alpha_7\)-integrin after vascular injury is a novel finding because the expression of \(\alpha_7\)-integrin is limited to differentiated muscle and is highly tissue specific (10, 52). Previous observations from Yao et al. (48) demonstrate that \(\alpha_7\) is ubiquitously expressed in various types of smooth muscle cells including vascular, gastrointestinal, and genitourinary cells. Certain VSMCs such as PAC1, R21969V9, and 9E11G (a cell line derived from P19 cells treated with retinoic acid), which maintain a stable expression of smooth muscle cell differentiation markers, also express a high level of \(\alpha_7\) in long-term culture (48). This is in agreement with our observation that \(\alpha_7\) is expressed in long-term VSMC cultures isolated from both control and AAM-injured rats.

However, in contrast with our studies that \(\alpha_7\)-integrin levels persist after serial passages of VSMCs, Yao et al. (48) noted a rapid loss of \(\alpha_7\)-integrin in dedifferentiated primary mouse VSMCs that have adapted to long-term culture conditions. Several experimental differences may account for the differences observed. In addition to the fact that different species were used in the two studies, the following experimental differences may also contribute to the differences seen: 1) In the study of Yao et al., primary mouse VSMCs were isolated by explant culture from the aorta. This method would yield predominately migratory VSMCs, whereas in our studies, VSMCs were isolated by enzymatic digestion such that a more heterogeneous population was isolated. 2) In Yao’s study, mouse primary cultures were grown on laminin-coated dishes, whereas in our study, VSMCs were placed directly on plastic culture dishes. 3) Different culture conditions were applied: for the mouse primary VSMCs, cells were cultured in \(\alpha\)-MEM with 7.5% FBS, whereas rat VSMCs in our study were cultured in M199 with 10% FBS. These differences and the conclusions drawn from them illustrate the need for more mechanistic and detailed studies of \(\alpha_7\)-integrin expression in VSMCs and in particular its role in vascular injury.
As shown in Fig. 6, A and B, α7-integrin staining was only present in the AAM-injured rats. However, because the antibody used for immunofluorescence imaging only recognizes the extracellular domain of α7-integrin, we performed immunoblot analysis using an antibody that recognizes the cytoplasmic domain of α7-integrin. Figure 7A shows an immunoblot of α7-integrin protein levels in aortas from four representative control and AAM-treated rats. The α7-integrin protein levels in control aortas were more variable than the higher levels observed consistently in AAM-treated aortas. Despite this variability, a significant increase in α7-integrin was demonstrated in AAM-injured rats (Fig. 7B), which confirms the results seen with immunofluorescence imaging. GAPDH was used as the loading control for the immunoblot analysis. The levels of GAPDH in control and AAM-treated animals did not correspond either positively or negatively to the levels of α7-integrin seen in the present study. This observation confirms that AAM increases the level of α7-integrin specifically.

Adhesion assays were performed to determine whether increased α7-integrin expression corresponded with functional alteration in VSMC adherence to laminin. As shown in Fig. 8, AAM VSMCs adhered to laminin more efficiently than control cells; this increase was attenuated by addition of anti-α7 antibody. These data show that increased α7-integrin expression in AAM VSMCs corresponds to increased adhesion to laminin. Partial attenuation of the AAM VSMC adhesion by anti-α7 antibody may be attributed to (1) the binding affinity of the specific antibody to α7-integrin and (2) the presence of other laminin-binding integrins such as αβ1, αβ2, and αβ3 that are also present in VSMCs and mediate cell adhesion and migration (4).

Based on the above observations, a putative functional role for α7-integrin in normal VSMCs and in response to vascular injury is intriguing; especially because its increased expression in muscular dystrophy and other forms of muscle injury has been documented. Previously, αβ1-integrin was shown to promote myoblast adhesion and migration on laminin-1 and laminin-2/4 (50) and mediate laminin-induced transmigration (49, 50) independent of the cell type (40). Thus increased α7-integrin expression during vascular injury may play a role in VSMC migration from the media to the intima during atherogenesis and reestablishment of basement membrane. Additionally, adhesion to laminin-2/4 promotes cell survival in myotubes (28). Thus increases in α7-integrin may contribute to both increased VSMC migration and survival during the atherogenic process.

In summary, our studies demonstrate for the first time that AAM increases α7-integrin expression in VSMCs and raises the possibility of its involvement in atherogenesis. Our observation of increased α7-integrin in chemically injured aortas also provides the first evidence of modulation of α7-integrin in response to vascular injury.

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DISCLOSURES

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