αV Integrins Are Necessary for Eutrophic Inward Remodeling of Small Arteries in Hypertension

Egidius H.J. Heerkens, Linda Shaw, Alisdair Ryding, Gillian Brooker, John J. Mullins, Clare Austin, Vasken Ohanian, Anthony M. Heagerty

Abstract—Human essential hypertension is characterized by eutrophic remodeling of small arteries, with little evidence of hypertrophy. Likewise, vessels of young hypertensive TGR(mRen2)27 animals have undergone similar structural alterations. The role of integrins in resistance arteries of TGR(mRen2)27 during the eutrophic-remodeling process was examined as blood pressure rose. Initially, 8 α and 3 β integrins were identified and levels of expression investigated using RT-PCR. As pressure increased and remodeling advanced, integrin expression profiles revealed that only αV was significantly raised. In conjunction, we confirmed elevated integrin αV protein levels in TGR(mRen2)27 rat arteries and localization to the media using immunofluorescence. β1 and β3, but not β5 integrin subunits were coprecipitated with integrin αV and are implicated in the eutrophic remodeling process. Administration of a peptide antagonist of αVβ3 abolished remodeling but enhanced growth, indicating that hypertrophy supervened as a response to hypertension-induced increases in wall stress. We have established that the only upregulated integrin, the αV subunit of integrin αVβ3, has a crucial role in the hypertensive remodeling process of TGR(mRen2)27 rat resistance arteries. During hypertensive remodeling, functions of specific αVβ3-extracellular matrix interactions are likely to allow vascular smooth muscle cell–length autoregulation, which includes a migratory process, to maintain a narrowed lumen after a prolonged constricted state. (Hypertension. 2006;47:281-287.)

Key Words: integrins ■ hypertrophy ■ hypertension ■ extracellular matrix

Although the causes of high blood pressure may vary, sustained hypertension is associated with changes in cardiovascular structure.1 These can be seen from the left ventricle down to the resistance vessels, and there is a strong relationship between left ventricular mass and small artery structure.2–3 Resistance to blood flow is provided by arteries with an internal diameter (ID) of ≈300 μm,4 and in subcortaneous vessels, there is clear evidence for an increased media thickness:lumen diameter ratio in essential hypertension, which is proportional to the severity of the blood pressure.1,5,6 Such findings appear to be representative of other vascular beds, with similar results being reported in the small intestine and the heart.7,8

The nature of the structural change that is effected by hypertension is a rearrangement of the preexisting material in the vascular wall without a major hypertrophic response.9 This has been termed eutrophic inward remodeling, which is initiated by vascular smooth muscle cell (VSMC)-length autoregulation.10 Constriction causes shortening of VSMCs and a return to normal length after dissipation of the stimulus; however, prolonged constriction causes cells to increase in overlap. This functional adaptation to prolonged vasoconstriction is thought to occur because it is an energetically favored mechanism to preserve a reduced lumen diameter for long periods.10 All models of hypertension appear to demonstrate eutrophic remodeling in small arteries, although to differing extents.1 Recently, small artery structural change has been reported to be more prognostically important than left ventricular hypertrophy.11

The TGR(mRen2)27 rat carries a DBA/2J Ren2 transgene12 and develops severe hypertension because of tissue renin–angiotensin activity.13,14 This model appears to display vascular wall changes, which approximate closely to those seen in essential or primary hypertension.15,16 Therefore, it provides a good model in which to investigate the cellular and molecular processes that are responsible for vascular remodeling.

In this context, in view of the lack of a major growth response, we examined the relationship between the extracellular matrix (ECM) and VSMC. The integrins are the major family of ECM receptors involved in mediating mechanotransduction from the extracellular environment into the cell and attachment and migration across many ECM molecules in vitro.17–19 In addition, it has been shown that protein translation is focused to sites containing integrins when tension is induced at points where cells adhere to surfaces in vitro.20

Received July 8, 2005; first decision July 25, 2005; revision accepted October 21, 2005.
From the Department of Medicine (E.H.J.H., L.S., C.A., V.O., A.M.H.), University of Manchester, Manchester Royal Infirmary, Manchester, United Kingdom; and Molecular Physiology Group A.R., G.B., J.J.M.), University of Edinburgh, Edinburgh, Midlothian, United Kingdom.
Correspondence to Anthony M. Heagerty, Department of Medicine, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL, United Kingdom. E-mail tony.heagerty@man.ac.uk
© 2006 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org

DOI: 10.116111.161101.HYP.0000198428.45132.02

281
Here we detail the examination of the expression of integrins in small arteries during the development of hypertension. The presence of all integrins was first established followed by a temporal investigation of upregulated integrins. The involvement of integrin αV then prompted us to examine which β-subunit was coprecipitated and which matrix ligands were present during eutrophic remodeling.

**Methods**

**Vessel Morphology**

Blood pressure of 4- to 8-week-old Sprague Dawley (SD) and tgr(mRen2)27 animals was monitored using indirect tail-cuff plethysmography under light halothane anesthesia. Rats were weighed and arteries were isolated from the proximal region of the mesentery, held in a physiological salt solution at 37°C, gassed with 5% CO2 and 95% O2, and mounted on a wire myograph. Subsequent morphological parameters of live vessels, such as media:lumen ratio, growth, and remodeling indexes, were then measured and calculated. Investigations were performed in accordance with the United Kingdom Home Office Regulated Procedures on Living Animals (Scientific Procedures) Act 1986.

**Competitive RT-PCR**

Identification of integrins was performed using sequence-specific primers, which were designed to produce PCR fragments ≤1000 bp. PCR products were compared with corresponding Genbank database sequences to confirm insert identity. Additional primer sequences and PCR conditions are available as an electronic supplement. Total DNA from arteries was extracted based on the method of Chomczynski and Sacchi, and competitive RT-PCR was performed and analyzed according to Siebert and Larrick. Quantitative data were expressed as the ratio of transcripts:hypoxantine guanine phosphoribosyl transferase transcripts.

**Integrin αV Antagonism**

At the age of 4 weeks, trgt(REN2)27 rats were injected intraperitoneally with cRGDfV peptide or cRADfV (10 mg/kg; Calbiochem) twice daily from day 28 for 5 days. On day 6, animals were euthanized and small arteries mounted on a wire myograph for morphological measurements.

**Integrin αV Western Analysis**

Anti-integrin αV antibody AB1930 (1:1000; Chemicon) was used in Western analysis according to Laemmli. Denitometric analysis was performed on a BioRad-GS690 scanner. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AB9485, Abcam) was used as a control for loading.

**Integrin αV Immunoprecipitation**

Immunoprecipitation of integrin αV was performed on 250-µg protein extract using antibody AB1930 and Aagarose-IgG/protein A (Sigma Aldrich) to identify the β subunit. Subsequent Western analysis was done using βf1 (AB1952, 1:1000), β3 (AB1932, 1:500), and β5 (AB1926, 1:100) integrin antibodies (Chemicon).

**Immunofluorescence Labeling**

Arteries were fixed in 4% paraformaldehyde-PBS at 4°C overnight, stored in 70% ethanol, paraffin embedded, and sliced at 4 µm. The anti-αV integrin (AB1930, 1:200) and nonimmune control IgG antibody was used followed by goat anti-rabbit antibody, Texas Red conjugated (Jackson Immunolabs), and diamidino-2-phenylindole (DAPI, Jackson Immunolabs) as a blue fluorescent nuclear stain. A Zeiss epiﬂuorescence-Axioplan2 microscope was used at ×400 magniﬁcation in conjunction with K300 software for image capture and analysis. Autofluorescence of the internal elastic lamina was visualized using a green ﬁlter set and was superimposed on rhodamine/DAPI fluorescence images to indicate the smooth muscle–endothelium boundary.

**Statistical Analysis**

The studies were performed on rats at between 4 and 8 weeks of age. Statistical analysis was done using MS Excel. Values are expressed as mean±SD. Differences between data were tested using 2-tailed unpaired homoscedastic Student t test or ANOVA for morphological measurements. P<0.05 was considered statistically significant. Linear regression curves for quantitative RT-PCR experiments were done using the Origin software package.

**Results**

**Blood Pressure**

A signiﬁcant rapid increase of blood pressure as a function of age was found in transgenic animals. Systolic blood pressure in TGR(mRen2)27 animals rose from 121±7.9 mm Hg at 4 weeks to 179±22.6 mm Hg at 8 weeks (P<0.001). Only small nonsignificant blood pressure rises in SD animals were observed (80.9±8.8 at 4 weeks to 93.4±8.13 mm Hg at 8 weeks).

**Vascular Morphology**

Mesenteric arteries (n=5; ID <300 µm) from the proximal region of the gut wall from TGR(mRen2)27 showed identical media:lumen ratios at 4 weeks when compared with SD (SD, 6.6±0.6%; tgr(mRen2)27, 6.45±0.8%). At 5 weeks, there was a significant increase in media:lumen ratio (1.4-fold) when compared with age-matched SD. Between 5 and 8 weeks, arteries of TGR(mRen2)27 animals retained a greater media:lumen ratio than controls (P<0.01), and media:lumen ratios of individual rat strains did not alter significantly after 5 weeks. Growth contributed significantly to structural changes of tgr(Ren2)27 arteries after 5 weeks: the media cross-sectional area was significantly increased in 8-week-old tgr(mRen2)27 rats (SD: 13482.5±1098; Ren2: 17659±1670 µm; P=0.0056; Figure 1A). Remodeling was prominent and complete in 5 weeks and older REN2 animals (90% to 87%) with growth indices ranging from 1% to 11% (Figure 1B) indicating that the major structural change is eutrophic remodeling, confirming a previous study.

**Integrin Quantitation**

PCR on reverse-transcribed RNA from small arteries revealed the presence of α1, α2, α3, α4, α5, α7, α8, αV, β1, β3, and β5 subunits (Figure 2A and 2B). The α7 and α5 (figure 2a) and β integrins (Figure 2B) were expressed at high levels in small arteries but were not significantly different between the 2 rat strains. However, the expression of integrin αV was increased significantly in TGR(mRen2)27 vessels compared with SD rats (P<0.05; n=6). As a consequence, the integrin αV subunit was examined from 4 to 8 weeks. Figure 3 demonstrates significant increases of expression of 2- to 2.9-fold at 5 (P<0.05; n=6), 6 (P<0.01; n=6), and 8 weeks (P<0.01; n=6) in vessels from TGR(mRen2)27.

Western analysis confirmed increases of integrin αV expression observed by RT-PCR and were 1.5-fold (P<0.05), 1.4-fold (P<0.05), and 2-fold (P<0.01), respectively, in 5-, 6-, and 8-week-old REN2 (n=5) arteries compared with controls (Figure 4B). Figure 4A is an example of a represen-
tative blot of integrin αV expression in tgr(REN)27 and SD arteries using antibody AB1930 (Chemicon). Relative integrin αV protein expression, established by densitometry, was corrected for loading by GAPDH and a standard control protein extract (% control) to correct for signal variability between blots (Figure 4A and 4B).

**Integrin αV Localization**

Immunofluorescence for integrin αV (AB1930, Chemicon) on 4-μm sections resulted in fluorescent staining of the medial layer and endothelial cells. Integrin αV fluorescence was more intense in medial layers of TGR(mRen2)27 rat arteries (Figure 5A) when compared with control SD animals (Figure 5C; n=4). Control incubations with nonimmune IgG did not result in any detectable signal (Figure 5B and 5D).

**Antagonism of Integrin αV**

Administration of an integrin αV antagonist (cRGDfV peptide, 10 mg/kg) in 4-week-old REN2 animals (n=4) resulted in a 1.3-fold reduction (P<0.005) of media:lumen ratios at 5 weeks. In addition, remodeling has changed from 97% in untreated to 9% in treated REN2 animals and accompanied by an increase of growth from 1% to 17% (Figure 6B). The media cross-sectional area was significantly increased (P<0.005) in arteries of cRGDfV-treated tgr(mRen2)27 animals (15606.6±11922) compared with control treatment (13164±1543; Figure 6A). However, systolic blood pressure was not significantly different between arteries of cRGDfV-treated and control REN2 animals (140.6±9.6 and 132±3.6 mm Hg, respectively).

**Immunoprecipitation of Integrin αV**

Using 5 μL of anti-integrin αV (AB1930) for 250 μg of arterial protein extract, we were able to coprecipitate integrin β1 (Figure 7A) and β3 (Figure 7B) but not β5 (Figure 7C) using nondenaturing conditions.

**Expression of Integrin αV Binding ECM**

As a result of our observation of increases of the integrin αV (β1/β3) subunit in TGR(mRen2)27 small arteries, it was decided to investigate the expression of ECM molecules that bind to this receptor. Smooth muscle cell–associated ECM components vitronectin, osteopontin and fibronectin were, therefore, investigated.
Expression of vitronectin was absent from small arteries, osteopontin was expressed at low levels in small arteries, and no differences in expression were observed using quantitative RT-PCR (data not shown). RT-PCR analysis identified fibronection and the splice form EIIIA (includes a 320-bp insert) in rat arteries, and the latter, although expressed at low levels, was significantly increased in tgr(REN)2 rat arteries at 6 (SD: 2.78 ± 0.33; REN2: 4.63 ± 1.16; P = 0.05) and 8 weeks (SD: 4.5 ± 0.6; REN2: 7.28 ± 0.93; P = 0.02; Figure 8). Western blotting confirmed the presence of fibronectin, but the EIIIA splice form was not detected in extracts of rat arteries.

Discussion
This study provides unequivocal evidence that a rise in blood pressure induces an adaptive vascular change in small arteries characterized as eutrophic inward remodeling. Furthermore, the process is brought about by an upregulation of Vimentin in the media, dimerizing with β3 and β1 integrins, which is likely to form a fibronectin receptor.

The choice of the TGR(mRen2)27 rat for these studies was based on previous reports that, as blood pressure develops, eutrophic remodeling occurs, and our results confirm this, with <12% of the morphological change in small arteries being attributable to growth. It is likely that VSMC hypertrophy is responsible for the increase in growth; however, it is
currently unknown whether it is accompanied by VSMC dedifferentiation and altered expression of contractile genes.

Initial expression profiles of vascular α1, α2, α3, α4, α5, α6, α7, α8, and αV integrin subunits revealed that only αV differed between the rat strains studied. The increase of integrin-αV expression was apparent at 5 weeks, increased additionally at 6 weeks, and appeared to be falling at 8 weeks of age. Western blotting for integrin αV confirmed the expression and increase in TGR(mRen2)27 arteries. Furthermore, it is clear from in situ hybridizations (data not shown) and immunofluorescent localizations that the predominant location for integrin αV subunit signals was the endothelium and the media.

There were near-significant increases in expression of α5, which has been reported to be elevated at the translational but not significantly at the transcriptional level in spontaneously hypertensive rat smooth muscle cells.25 The preference of hypertensive TGR(mRen2)27 arterial smooth muscle cells to upregulate integrin αV above others could be explained by its role of replacing other integrins, because integrin αV is known to substitute fibronectin-dependent functions in vivo in α5β1-deficient mice.26

The relevance of the increase in the integrin αV in hypertension could also be its involvement in mechanotransduction from the extracellular environment into the cell.17 In addition, it was shown that αVβ3, but not β1, integrins colocalized near VSMC focal contact sites during migration15,27 implicating β3 integrins also in signal transduction.

By intraperitoneal administration of a specific αVβ3 antagonist, cRGDFV, we abolished remodeling, indicating that this receptor is responsible for hypertension-mediated eutrophic remodeling. In addition, an increase of the media cross-sectional area was observed in treated animals, indicating that growth supervened. We used the cRGDFV peptide because it was shown to be inhibitory for αVβ3 (IC50 = 50 μM) matrix binding and used for rodent cells.28,29 Also, other components, such as blocking antibodies, have been shown to cause vasodilation,30,31 which could have confounded our studies.

In our experiments, we identified the integrin αVβ3 heterodimer as responsible for these arterial structural changes, because β3 and β1 integrin subunits were coprecipitated with αV. Furthermore, inhibition of remodeling observed with the specific αVβ3 antagonist cRGDFV is almost complete and suggests a significant role for αVβ3 in migration of smooth muscle cells in hypertension-mediated arterial remodeling.

The function of αVβ1 present on VSMCs of resistance arteries is not entirely clear, but it could be hypothesized that the role of this integrin is more in VSMC attachment than migration.17,18,27 The event that initiates upregulation of αV integrins, VSMC migration, and subsequent eutrophic remod-
elings are not clear. However, evidence suggest that the onset of this process is evoked by enduring $\alpha_{1b}$-adrenergic receptor activation, independent of blood pressure,\textsuperscript{32} which results in prolonged constriction and VSMC length autoregulation.\textsuperscript{10}

In conjunction with integrin $\alpha V \beta 3$ subunit upregulation in TGR(mRen2)27 small arteries, we quantitated 3 ECM ligands known to bind $\alpha V \beta 3$. Of investigated ECM components, vitronectin was not detected in the vascular matrix, and osteopontin, although present at low levels, did not change in TGR(mRen2)27 arteries. The soluble isoform of fibronectin was abundantly expressed but did not change in TGR(mRen2)27 arteries. The mRNA expression of splice variant fibronectin EIIIA+, however, was raised in TGR(mRen2)27 rat arteries at 6 and 8 weeks. This isoform has been reported to be upregulated in VSMCs of the aorta of deoxycorticosterone-salt-hypertensive rats\textsuperscript{33}. In addition, it has also been shown to facilitate the incorporation of EIIA+ into the existing ECM.\textsuperscript{34} We speculate that expression and incorporation of (by Western blotting undetectable) small amounts of fibronectin EIIIA+ in an existing matrix is to create their own microenvironment for accurate signal transduction and phenotypic function.\textsuperscript{35} All of the above evidence indicates that the most likely candidate to form interactions with integrin $\alpha V \beta 3$ is fibronectin. However, because of the promiscuous nature of $\alpha V \beta 3$, we cannot rule out the effect of other ECM components on inward eutrophic remodeling: many exotic ECM-proteins not covered in this study contain an RGD motive and could additionally influence VSMC migration and attachment during remodeling. In addition, matrix proteins can be modified directly at the integrin binding site: ECM forms a substrate for factors, such as tissue-type transglutaminase, which coassociates with integrins to bind fibronectin\textsuperscript{36} to potentially alter matrix constituents by cross-linking. Furthermore, it is suggested that tissue-type transglutaminase present in resistance arteries fixates specific ECM constituents after a prolonged state of vasoconstriction, and it can be hypothesized that ($\alpha V$) integrins play a role in the regulation of this process.\textsuperscript{37}

**Perspectives**

We have demonstrated that $\alpha V \beta 3$ integrins are necessary for hypertension-associated eutrophic remodeling. How eutrophic remodeling is overwhelmed by severe hypertension and replaced by deleterious hypertrophy must be the next research target, because such changes at the level of the small arteries are often observed in patients with fulminant or accelerated forms of hypertension\textsuperscript{38}; homeostatic mechanisms that occur in response to a rise in blood pressure are overwhelmed, and when these break down, a growth response ensues, and this is prognostically adverse. Hypertrophic changes in the vascular wall are also observed in diabetes irrespective of whether hypertension is present or not. Such patients are prone to downstream target organ damage and have defective autoregulatory processes.\textsuperscript{39} It is tempting to speculate that the development of hypertrophy is prognostically adverse and identifies patients at risk of developing hypertension-associated complications.

**Acknowledgments**

This study was supported by the British Heart Foundation. We thank Maureen Speed for expert secretarial assistance. The Wellcome Trust funded A.R. J.J.M. is a Wellcome Trust Principal Fellow.

**References**


αV Integrins Are Necessary for Eutrophic Inward Remodeling of Small Arteries in Hypertension

Egidius H.J. Heerkens, Linda Shaw, Alisdair Ryding, Gillian Brooker, John J. Mullins, Clare Austin, Vasken Ohanian and Anthony M. Heagerty

Hypertension. 2006;47:281-287; originally published online December 27, 2005;
doi: 10.1161/01.HYP.0000198428.45132.02

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/47/2/281

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2006/01/30/01.HYP.0000198428.45132.02.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/