Cardiovascular Pathology, Vol. 13(2), 79-84, 2004

Expression of coxsackievirus and adenovirus receptor (CAR) in neointima of the rat carotid artery

Akimitsu Nasuno,[1] Ken Toba,[1] Takuya Ozawa, [1] Haruo Hanawa,[1] Yasser Osman, [1] Yuko Hotta,[2] Kaori Yoshida,[1] Takashi Saigawa,[1] Kiminori Kato, [1] Ryozo Kuwano[2], Kenichi Watanabe[3], and Yoshifusa Aizawa[1]

[1]Divisions of Cardiology and Hematology, and [2]Research Laboratory for Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
[3]Department of Clinical Pharmacology, Niigata College of Pharmacy, Niigata, Japan

Corresponding author: Ken Toba, M.D., Division of Hematology, Niigata University Graduate School of Medical and Dental Sciences, 1-754 Asahimachi-dori, Niigata, 951-8510, Japan.Tel: +(81)-25-227-2185; Fax: +(81)-25-227-0774; e-mail: tobaken@med.niigata-u.ac.jp

Abstract Our previous study revealed that the coxsackievirus and adenovirus receptor (CAR) is a homophilic cell adhesion molecule and may function as a sensor of cell-cell interactions in the brain and damaged heart. In this study, we investigated if CAR expression is involved in the formation of neointimal hyperplasia using a balloon injury model of rat carotid artery. Cultured vascular smooth muscle cells (SMCs) from rat aorta were also studied.

CAR antigen was constitutively detected in the endothelial cells (ECs) but not in SMCs before injury. On day 5 after balloon injury, CAR was expressed strongly in the first layer of medial SMCs. Neointimal hyperplasia was observed on day 7, and strong expressions of CAR concomitantly with proliferating cell nuclear antigen (PCNA) were obvious in the neointimal SMCs, while CAR in medial SMCs disappeared. The expression of CAR mRNA reached a peak on day 7 and declined gradually to the basal levels. When the ECs regenerated on day 14, CAR antigen was observed in the ECs but disappeared in the neointima. CAR together with PCNA was expressed abundantly in the proliferating SMCs in vitro, and diminished in cells grown to a confluent state. The abundant expression of CAR in the neointima may facilitate an adenoviral gene therapy.

Keywords: Coxsackievirus and Adenovirus receptor (CAR), intimal hyperplasia, vascular smooth muscle cells, balloon injury

Introduction

A high-affinity receptor for coxsackievirus and adenovirus types 2 and 5, CAR, has recently been identified as a 46-kDa transmembrane cell adhesion molecule, which belongs structurally to the immunoglobulin superfamily containing VCAM-1 and ICAM-1.[1-3] The physiological functions of CAR are not well understood. A previous study revealed that CAR is a homophilic cell adhesion molecule and was expressed strongly in the brain and heart of embryonic mice until the neonatal phase[4] suggesting that this molecule may play a role in the adhesion of embryonic cells during the development of these organs.

Although CAR molecule has been shown to be abundant in the hearts of newborn but not in adult rats, CAR molecule was found to be re-expressed strongly in the damaged rat heart: during the active phase of experimental autoimmune myocarditis.[5] This transient nature of CAR expression might suggest a role of the adhesion molecule in sensing the state of the cell-to-cell contact. When cell-to-cell contact is loosened, it may be expressed until the repair or organization is completed. Although the role of CAR in the vascular system is not known, like in damaged cardiomyocytes, CAR molecule might be involved in the regeneration of the damaged vascular system.

In the present study, CAR expression was investigated in an experimental model of intimal hyperplasia and in cultured vascular smooth muscle cells (SMCs). If CAR is expressed in the injured artery or the neointimal hyperplasia, CAR may explain the efficient adenovirus-mediated gene transfer to the neointima and to a sclerotic plaque as reported previously.[6,7] The physiological and pathological roles of CAR in injured vessels and in intimal hyperplasia should be elucidated further.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (250–290 g) were obtained from Charles River Japan Inc. (Yokohama, Japan). All procedures were performed under sterile conditions with the

approval of the Institutional Animal Care and Use Committee in compliance with procedures and methods outlined by the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23; National Institutes of Health, Bethesda, MD).

Balloon injury and sampling

Rats were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). Carotid arteries were denuded of endothelium by the introduction of a 2F Fogarty balloon embolectomy catheter (Baxter Healthcare, Santa Ana, CA) into the left common carotid artery through the external carotid. The balloon was inflated to distend the common carotid artery and then withdrawn to the external carotid artery. This procedure was repeated three times. After removal of the catheter, the external carotid artery was ligated and the wound closed.

The rats were sacrificed on days 3, 5, 7, 10, 14 and 21 after treatment, and the injured left carotid arteries were obtained by isolation from the surrounding tissue. Control untreated rats were also studied for comparisons.

Antibodies, tissue preparation and immunohistochemistry

A short polypeptide of 17 amino acid residues (KTQYNQVPSEDFERAPQ) in the intracellular domain of murine CAR was synthesized. Rabbit anti-CAR antiserum was obtained by immunization of white rabbits with the peptide and the immunoglobulin fraction was partially purified and used for immunohistochemistry as described previously.[8] This antibody also reacts with rat CAR.[5]

After excision and washing of the carotid, the samples were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen in liquid nitrogen and stored at –80°C. Serial frozen sections (thickness, 7 μm) were applied to APS-coated slides (Matsunami Glass, Osaka, Japan), fixed in cold Zamboni liquid for 30 min, and then stained with rabbit polyclonal antibodies against CAR, factor VIII-related antigen (FVIIIRAg; Zymed, San Francisco, CA), and a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, Zymed). Rabbit antibodies were detected with biotinylated goat anti-rabbit Ig (Nichirei, Tokyo, Japan) and FITC-conjugated streptavidin (Vector, Burlingame, CA), and the mouse monoclonal antibody was detected with a rhodamine-conjugated goat anti-mouse secondary antibody (Vector). Sections were also stained with control rabbit IgG and propidium iodide (PI) to estimate nonspecific binding and to stain nuclei, respectively.

Real-time quantitative RT-PCR

Total RNA was isolated from the carotid samples with Trizol (Life Technologies, Tokyo, Japan). cDNA was synthesized from 2 μg total RNA with a random primer and a RNA PCR Kit (AMV) Ver. 2.1 (Takara, Tokyo, Japan) in a final volume of 20 μL. Templates of CAR and γ-actin were made as a control gene to calculate the standard concentration curve. cDNA from the samples was amplified with each primer (CAR-5': GAACAGAGGATCGAAAAAGCTAAAG and CAR-3': TCGTTACTCGTAAAGTGTACTCGTC,[9] γ-actin-5': AGCCTTCCTTCCTGGGCATGGAGT and γ-actin-3': TGGAGGGGCCTGACTCGTCATACT[10]), and inserted directly into the pGEN-T vector. The recombinant plasmid was then isolated after transforming into *Escherichia coli* JM109 competent cells. cDNA was diluted 100-fold with DNAse-free water, and 5 μL of the sample was used for CAR and γ-actin real-time quantitative PCR. The cDNA and diluted recombinant plasmid were amplified with the primers and dye (LightCycler Fast Start DNA Master SYBR Green I; Roche, Indianapolis, IN). After initial denaturation for 10 min at 94°C, a 3-step cycle procedure was used (denaturation at 94°C for 1 min, annealing at 58°C for 1.5 min, and extension and acquisition at 73°C for 2 min, for 35 cycles). A standard curve was calculated using LightCycler software, and drawn by plotting the cycle number at which the fluorescent signals entered the log-linear phase against the concentration of the standards. The relative quantities of CAR transcripts in all samples were calculated using LightCycler software and the standard curve. The final result was the normalized CAR value, expressed as the ratio of CAR/γ-actin.

Culture of vascular SMCs

Rat aortic vascular SMCs were purchased from Cell Applications, San Diego, CA. The cells were washed and suspended in a smooth muscle cell proliferation medium (SMC P-STIM, Becton

Dickinson: MCDB131 medium supplemented with 5% fetal bovine serum, 5 μg/L of human recombinant insulin, 10 μg/L of human recombinant EGF, 2 μg/L of human recombinant bFGF, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate). Cells (1×10^4) in 0.7 ml medium were seeded in a collagen type-1 coated culture slide (Biocoat, Becton Dickinson: 8 wells/slide, 0.69 cm^2/well), and incubated at 37°C in 5% CO_2 in humidified air for 1 to 4 days. Culture slides were harvested, washed with PBS, fixed as described above, and stained for CAR and PCNA antigens. CAR was stained by serial incubation with rabbit anti-CAR, biotinylated goat anti-rabbit Ig and FITC-conjugated streptavidin. PCNA was stained using mouse anti-PCNA and TRITC-conjugated goat anti-mouse IgG. Rabbit IgG was used as a negative control to detect background fluorescence.

Confocal Microscopy

Fluorescence was detected with a microscope (IX71, Olympus, Tokyo, Japan), a confocal laser scan unit (FV500, Olympus), and a Fluoview software (version 4.0, Olympus). Fluorescent probes were excited with a multi-argon laser (488nm for FITC, Rhodamine and PI) and a Green He-Ne Laser (543nm for TRITC). Fluorescence was detected with the barrier filter sets, BA505/525 (FITC), BA560IF (Rhodamine and TRITC), and BA610IF (PI), and assigned as green (FITC) and red (Rhodamine, TRITC and PI). Two-color images were obtained by overlaying images from individual channels acquired from a sample. Graphic files obtained by confocal microscopy were analyzed using NIH-Image software to estimate CAR expression. Green light brightness was measured in vessel lumen (background noise), intima, and media. Relative mean fluorescence intensity (rMFI) was utilized as representative of quantity of CAR antigen: (green brightness of a tissue region) / (green brightness of vessel lumen).

Statistical analysis

Means and standard deviation (SD) were calculated and used to express the mRNA levels. Comparisons of the values among groups were performed by one-way ANOVA followed by the Bonferroni multiple comparison test. Paired *t*-test was used to compare rMFI in each tissue regions in 3 samples.

Results

Expression of CAR and PCNA antigens

Carotid artery samples obtained at least 3 (3 to 5) rats were immunohistochemically analyzed at each time points after balloon injury treatment. Figure 1 shows CAR and PCNA antigen expression along with the FVIIIRAg-stain in endothelial cells (ECs) using serial sections of the carotid artery. The normal carotid of untreated rats had a recognizable endothelial monolayer and medial layers. CAR-antigen was positive in the ECs, and absent or only trace-level positive in the media. Regeneration of ECs and thickening of intimal SMCs were not obvious on day 5 of treatment. However, intensive expression of CAR antigen was observed in superficial medial SMCs at this time. Regenerating ECs and neointima were evident on day 7, and both ECs and neointimal SMCs strongly expressed CAR antigen, while CAR antigen observed in medial SMCs on day 5 clearly decreased on day 7. CAR antigen expression was limited to the new EC layer on day 14. The neointimal SMCs observed on day 7 also expressed PCNA in the nucleus, indicating a high degree of cell proliferation similar to that of embryonic SMCs.[11] Measured rMFI of CAR antigen (n=3) was as follows: 6.36 $\pm$ 0.42 in ECs and 1.49 $\pm$ 0.07 in media ($p < 0.01$) in untreated rats; 11.65 $\pm$ 1.78 in superficial medial SMCs and 2.76 $\pm$ 0.65 in outer media ($p < 0.05$) on day 5; 7.95 $\pm$ 0.58 in neointimal SMCs and 1.39 $\pm$ 0.02 in media ($p < 0.01$) on day 7; and 10.27 $\pm$ 1.45 in regenerated ECs, 2.89 $\pm$ 0.18 in neointima ($p < 0.05$), and 2.90 $\pm$ 0.26 in media ($p < 0.05$) on day 14.

Expression level of CAR mRNA

The time-course of the CAR mRNA level in the injured carotid artery is shown in Figure 2. The means ± SD of the ratio of CAR/γ-actin mRNA were as follows: 0.072 ± 0.077 (n = 8) in carotid arteries of untreated rats; 0.075 ± 0.060 (n = 5) in treated carotids on day 3; 0.124 ± 0.072 (n = 7) on day 5; 0.180 ± 0.109 (n = 8) on day 7; 0.152 ± 0.065 (n = 7) on day 10; 0.135 ± 0.022 (n = 5) on day 14; and 0.122 ± 0.036 (n = 6) on day 21. Carotid arteries obtained from untreated rats expressed low levels of CAR mRNA. This level increased to a maximum on day 7 ($p = 0.0050$), and then decreased gradually to a low level thereafter.

Cultured SMCs

Proliferating vascular SMCs in culture

expressed CAR and PCNA in the cytoplasm and nucleus, respectively, therefore the cells actively produced CAR antigens in the growing stage (Figure 3). CAR antigen clearly diminished in the cells grown to a confluent stage. Artifact gap was seen among the confluent cells by fixation, which may indicate the cell-cell contact of SMCs was not tight. The same experiment was carried 4 times.

Discussion

In untreated carotid arteries, CAR antigen was constitutively expressed in ECs and the baseline level of CAR mRNA was thought to have arisen from these cells. Denudation of the media by stripping the EC layer off the carotid artery caused the induction of CAR in the medial SMCs before formation of the neointima, and this resulted in increased levels of CAR mRNA. Therefore, upregulation of CAR might have been triggered by the loss of cell-to-cell contact through mechanical injury, and restoration of the injured carotid occurred as a result. Several days after the treatment, neointimal SMCs and a newly generated EC layer appeared with strong expression of CAR. CAR expression in the injured carotid neointima was accompanied by strong expression of PCNA, and both antigens then decreased simultaneously. As transient expression of adhesion molecules is thought to be closely related to the development of intimal hyperplasia,[12,13] CAR expression may be another trigger of these physical and pathological processes in the arteries.

So far, adenovirus-mediated gene transfer has been reported to accumulate in injured vessels, and the efficacy of this transfection is reported to be higher than liposomal or retroviral-mediated gene delivery.[14,15] The time sequence of CAR expression in the present study appeared to be almost the same as the time-course of integration of a recombinant adenovirus (AdV) containing β-galactosidase in a balloon injury model of the rat carotid artery reported in an earlier work[1], i.e., AdV integrated into ECs before balloon treatment, accumulation was scattered in the medial layer before formation of the neointima, and massive integration of the virus was observed in neointimal hyperplasia. AdV integration was also observed in intimal macrophages and SMCs in human vessels.[2] VCAM-1 shares some homology with CAR and is thought to contribute to the integration of adenovirus vectors into vessels.[16] The present study revealed that the high-affinity receptor of AdV, CAR itself, was expressed in the EC layer and in the neointima thereafter. Hence, CAR itself, but not VCAM-1, may play an essential role in adenovirus vector integration into the vessels.

AdV most likely utilizes CAR and vitronectin receptors (VNRs), αVβ3 and αVβ5, as primary attachment site for the adenovirus fiber[2,3] and as secondary internalization receptors,[17,18] respectively. VNR-αVβ3 is expressed in ECs and monocytes, while αVβ5 is expressed in fibroblasts, platelets, epithelial cells, and SMCs. On the other hand, CAR expression in vascular cells is not established so far. Cultured prostate SMCs expressed CD51 (integrin αV), but not CAR, in the cell surface analyzed by flow cytometry, and AdV infected into SMCs at a very low frequency.[19] However, like ECs, SMCs may also show different phenotypes in individual tissues and organs. In a report of *ex vivo* AdV gene transfer into aortas, AdV-mediated LacZ expression was abundant in endothelium and adventitial cells, and medial SMCs also expressed LacZ at a low frequency.[20] Therefore, some level of CAR is possibly expressed in vascular SMCs. In fact, AdV transduction via integrin receptors was strikingly suppressed when CAR binding sites were blocked in cultured human aortic SMCs.[21] In the present study, CAR was expressed abundantly in the cytoplasm of cultured vascular SMCs, and membrane-bound CAR was not perceived by the huge fluorescence scattering of cytoplasmic FITC. However, we supposed that CAR should be expressed in the cell surface in a low level because of the nature of CAR molecule, i.e., type I membrane glycoprotein to function as cell adhesion molecule.

The role of CAR has been studied in virus infection, vascular ECs as well as in epithelial cells in airway and bladder cancer. CAR mediated homotypic intercellular contact, recruited ZO-1 (a tight junction protein) at the tight junction, and constituted the functional barrier in tracheobronchial epithelial cells as a result.[22] Interestingly, the ligand for CAR is thought to be CAR itself, because specific antibodies against the extracellular domain of CAR and recombinant soluble CAR inhibited the homotypic cell aggregation.[4,22] On the other hand, CAR expression in human umbilical vein ECs increased with culture confluence.[23] Moreover, cell-cell contact initiated by CAR inhibited the in vitro growth of bladder cancer cells accompanied by p21 and hypophosphorylated retinoblastoma

protein accumulation.[24] Taken together, the upregulated CAR expression in the regenerated ECs might have inhibited and terminated the growth of SMCs through CAR-CAR interaction expressed in the SMCs in our study.

Little is known about the role of CAR in the pathogenesis of cardiovascular disorders. We previously reported that CAR was expressed in the heart of rats with experimental autoimmune myocarditis,[5] and this observation may in part explain the susceptibility of coxsackievirus-mediated myocarditis in the damaged heart. CAR is also found to be upregulated in the human heart with dilated cardiomyopathy.[25] The precise role of CAR in cardiovascular disease needs to be determined. In conclusion, CAR was abundantly expressed in the intimal hyperplasia. Such abundant expression of CAR molecules may have clinical significance especially in adenovirus-mediated gene therapy to prevent post-PTCA restenosis. As in vivo administration of an antibody cocktail against ICAM-1 and LFA-1 inhibited intimal hyperplasia in rats,[12] the CAR antigen may also be a potential target molecule for antibody therapy in vivo. The physiological and pathological roles of CAR in injured vessels and in intimal hyperplasia should be elucidated further.

Acknowledgement

This work was supported by a Grant for Research on Specific Disease from the Japanese Ministry of Health, Labor and Welfare.

References

1. Bergelson JM, Krithivas A, Celi L, Droguett G, Horwitz MS, Wickham T, Crowell RL, Finberg RW: The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J Virol.* 1998;72: 415-419.
2. Tomko RP, Xu R, Philipson L: HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci USA.* 1997;94:3352-3356.
3. Bergeson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW: Isolation of a common receptor for coxsackie B virus and adenovirus 2 and 5. *Science.* 1997;275:1320-1323.
4. Honda T, Saitoh H, Masuko M, Katagiri-Abe T, Tominaga K, Kozakai I, Kobayashi K, Kumanishi T, Watanabe YG, Odani S, Kuwano R: The coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. *Molecular Brain Research* 2000;77:19-28.
5. Ito M, Kodama M, Masuko M, Yamaura M, Fuse K, Uesugi Y, Hirono S, Okura Y, Kato K, Hotta Y, Honda T, Kuwano R, Aizawa Y. Expression of coxsakievirus and adenovirus receptor in hearts of rats with experimental autoimmune myocarditis. *Circ Res.* 2000;86:275-280.
6. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T: Efficient and selective adenovirus-mediated gene transfer into vascular neointima. *Circulation.* 1993; 88:2838-2848.
7. Rekhter MD, Simari RD, Work CW, Nabel GJ, Nabel EG, Gordon D: Gene transfer into normal and atherosclerotic human blood vessels. *Circ Res.* 1998;82: 1243-1252.
8. Abe TK, Tanaka H, Iwanaga T, Odai S, Kuwano R. The presence of the 50-kDa subunit of dynactin complex in the nerve growth cone. *Biochem Biophys Res Commun.* 1997;233:295-299.
9. Fechner H, Haack A, Wang X, Eizema K, Pauschinger M, Schoemaker R, Veghel R, Houtsmuller A, Schultheiss HP, Lamers J, Poller W: Expression of coxsackie adenovirus receptor and alphav-integrin does not correlate with adenovector targeting in vivo indicating anatomical vector barriers. *Gene Ther.* 1999;9:1520-35.
10. Brown CW, McHugh KM, Lessard JL. A cDNA sequence encoding cytoskeletal gammer-actin from rat. *Nucleic Acids Res.* 1990;17:5312.
11. Asahara T, Bauters C, Pastore C, Kearney M, Rossow S, Bunting S, Ferrara N, Symes JF, Isner JM. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation.* 1995;91:2793-2801.
12. Yasukawa H, Imaizumi T, Matsuoka H, Nakashima A, Morimatsu M: Inhibition of intimal hyperplasia after balloon injury by antibodies to intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1. *Circulation.* 1997;95:1515-1522.
13. Barron MK, Lake S, Buda AJ, Tenaglia AN: Intimal hyperplasia after balloon injury is attenuated by blocking selectins. *Circulation.*

1997;96:3587-3592.
14. Leclerc G, Gal D, Takeshita S, Nikol S, Weir L, Isner JM: Percutaneous arterial gene transfer in a rabbit model: efficiency in normal and balloon-dilated atherosclerotic arteries. *J Clin Invest.* 1992;90:936-944.
15. Flugelman MY, Jaklitsch MT, Newman KD, Cassells W, Bratthauer GL, Dichek DA: Low level in vivo gene transfer into the arterial wall through a perforated balloon catheter. *Circulation*. 1992;85:1110-1117.
16. Chu Y, Heistad DD, Cybulsky MI, Davidson BL: Vascular cell adhesion molecule-1 augments adenovirus-mediated gene transfer. *Arteriorscler Thromb Vasc Biol.* 2001;21:238-242.
17. Wickham TJ, Filardo EJ, Cheresh DA and Nemerow GR: Integrin $\alpha_v\beta_5$ selectively promotes adenovirus mediated cell membrane permeabilization. *J Cell Biol*. 1994; 127: 257-264
18. Chiu CY, Mathias P, Nemerow GR and Stewart PL: Structure of adenovirus complexed with its internalization receptor, $\alpha_v\beta_5$ integrin. *J Virol*. 1999; 73: 6759-6768.
19. Kirkman W, Chen P, Schroeder R, Feneley MR, Rodriguez R, Wickham TJ, King CR, and Bruder JT: Transduction and apoptosis induction in the rat prostate, using adenovirus vectors. *Hum Gene Ther*. 2001; 12: 1499-1512.
20. Suzuki M, Ishizaka N, Hwang WM, Tsukamoto K, Minami K, Aizawa T, Mori I, Taguchi J, Nagai R, and Ohno M. Application of hydrostatic pressure facilitates *ex vivo* adenovirus gene transfer into rabbit aortas. Biochem Bioph Res Co. 2000; 274: 422-426.
21. Biermann V, Volpers C, Hussmann S, Stock A, Kewes H, Schiedner G, Herrmann A, and Kochanek S. Targeting of high-capacity adenoviral vectors. *Hum Gene Ther*. 2001; 12: 1757-1769.
22. Cohen CJ, Shieh JTC, Pickles RJ, Okegawa T, Hsieh J-T, Bergelson JM: The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci USA*. 2001;98:15191-15196.
23. Carson SD, Hobbs JT, Tracy SM, Chapman NM: Expression of the coxsackievirus and adenovirus receptor in cultured human umbilical vein endothelial cells: regulation in response to cell density. *J Virol*. 1999;73: 7077-7079.
24. Okegawa T, Pong R-C, Li Y, Bergelson JM, Sagalowsky AI, Hsieh J-T: The mechanism of the growth-inhibitory effect of coxsackievirus and adenovirus receptor (CAR) on human bladder cancer: a functional analysis of CAR protein structure. *Cancer Res*. 2001;61:6592-6600.
25. Noutsias M, Fechner H, de Jonge H, Wang X, Dekkers D, Houtsmuller AB, Pauschinger M, Bergelson J, Warraich R, Yacob M, Hetzer R, Lamers J, Schultheiss HP, Poller W: Human coxsackie-adenovirus receptor is colocalized with integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the cardiomyocyte sarcolemma and upregulated in dilated cardiomyopathy. *Circulation.* 2001;104:275-280.

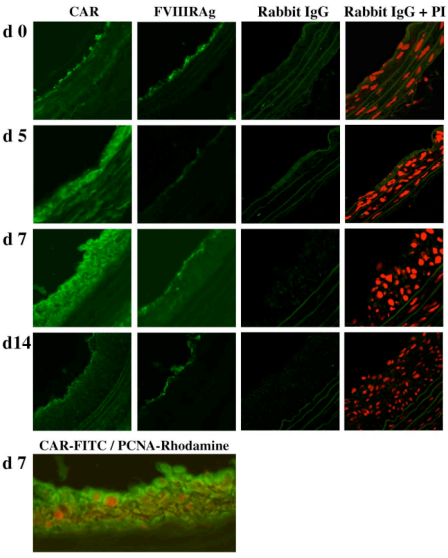

Fig. 1. Distributions of CAR and PCNA antigens after balloon injury to the rat carotid artery. CAR antigen (FITC), factor VIII-related antigen (FITC), rabbit IgG (controls for CAR, FITC) and nuclear staining (PI) are demonstrated, and two-color staining of CAR and PCNA (rhodamine red) is also shown in the bottom panel. CAR was constitutively expressed in vascular endothelial cells (day 0), and was induced in the first layer of medial SMCs on day 5. The antigen disappeared from medial SMCs, and was strongly expressed in the neointimal SMCs simultaneously with proliferating cell nuclear antigen (PCNA) on day 7. It vanished from the vascular SMC after the regeneration of EC. Photographs are representatives of carotid samples obtained from at least 3 different rats at each time points after treatment. Original magnification, x 400 (days 0, 5 and 7); x 200 (day 14); and x 800 (two-color).

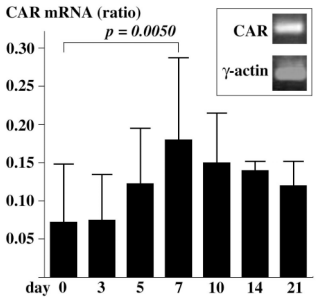

Fig. 2. Time-course of the level of expressed CAR mRNA after balloon injury to the rat carotid artery (mean ± SD). Vertical axis, ratio of measured mRNA, CAR/γ-actin, by real-time quantitative RT-PCR. *p*, compared with control carotid artery of untreated rats (day 0) .

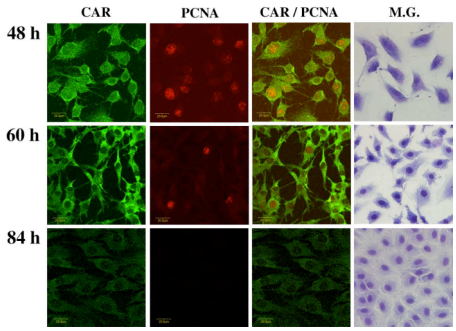

Fig. 3. Morphology and antigen expressions of CAR and PCNA in cultured vascular SMCs. Proliferating SMCs expressed CAR (FITC, green) and PCNA (TRITC, red) antigens (48 and 60 hours). The cells grown to a confluent state lost antigens of PCNA, and CAR expression clearly diminished in the cells (confocal laser scan system). Morphologies of the cultured cells in different fields are also shown (May-Giemsa stain). Note that artifact gap was seen in the cells grown to a confluent stage by fixation of the sample. Four different cell cultures were carried, and representative photographs are demonstrated.