Histol Histopathol (2015) 30: 233-244
DOI: 10.14670/HH-30.233

**http://www.hh.um.es**

**Histology and Histopathology**
*Cellular and Molecular Biology*

# Endoglin is not expressed with cell adhesion molecules in aorta during atherogenesis in apoE-deficient mice

**Jana Rathouska*, Katerina Jezkova*, Ivana Nemeckova, Lenka Zemankova, Michala Varejckova and Petr Nachtigal**
Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Prague, Czech Republic
*These authors contributed equally to this work

**Summary.** Endoglin (TGF-β receptor III), has been demonstrated to affect vascular endothelium and atherosclerosis. Moreover, it was also demonstrated that endoglin is involved in inflammation and plays a role in leukocyte adhesion and transmigration *in vitro* and *in vivo* but not in atherosclerosis related vessels. In this study, we wanted to evaluate endoglin expression in two different parts of the aorta (heart aortic sinus and ascending aorta) and assess its potential simultaneous expression with cell adhesion molecules in non-atherosclerotic and atherosclerotic aortas of apoE-deficient mice.

Ten-week–old female apolipoprotein E-deficient mice on a C57BL/6J background (n=24) were randomly subdivided into three groups and were fed either chow diet (for another two months) or Western type diet (for another two or four months). Immunohistochemical staining of endoglin, VCAM-1 and P-selectin in aortic sinus and ascending aorta was performed.

Endoglin expression was detected only in endothelial cells and varied during atherogenic process in aorta but not in aortic sinus. Moreover, its expression seemed to be weaker in aorta when compared to aortic sinus and the positivity was detected only in endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium regardless of the plaque size. Endoglin was not expressed with P-selectin and VCAM-1 in aortic endothelium in any studied group.

This study shows that endothelial expression of endoglin is related to the atherogenic process predominantly in aorta outside the heart. Moreover, endoglin is not localized with cell adhesion molecules involved in atherosclerosis, suggesting it might not participate in leukocyte accumulation in aorta of apoE-deficient mice during atherogenesis.

**Key words:** Endoglin, P-selectin, VCAM-1, Atherogenesis, apoE-deficient mice

*Offprint requests to*: Nachtigal Petr, Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, Hradec Kralove 500 05, Czesch Republic. e-mail: Petr.Nachtigal@faf.cuni.cz

## Introduction

Endoglin, an accessory receptor of the TGF-β signaling pathway is a homodimeric transmembrane glycoprotein that has been demonstrated to play a role in vascular physiology and pathology (Lopez-Novoa and Bernabeu, 2010). Changes of endoglin expression and function were detected in hereditary hemorrhagic telangiectasia, preeclampsia, hypertension, cancer and atherosclerosis (Levine et al., 2006; ten Dijke et al., 2008; Perez-Gomez et al., 2010; Nachtigal et al., 2012).

Endoglin expression was detected in atherosclerosis prone vessels in both humans and experimental animals. Endothelial cells (Nachtigal et al., 2009a), smooth muscle cells (Conley et al., 2000) and even macrophages (Piao and Tokunaga, 2006) are able to express endoglin in different stages of the atherosclerotic process. In addition, endoglin is able to affect the expression and activity of endothelial nitric oxide synthase (eNOS), including vasodilatation/vasoconstriction of arteries (Jerkic et al., 2004), production of collagen in vessels by

smooth muscle cells (Bot et al., 2009) and formation of new blood vessels in atherosclerotic plaques (Luque et al., 2009). The above-mentioned effects of endoglin might affect atherosclerosis differently and therefore a precise role of endoglin in this process has not been fully elucidated so far.

More recently, Rossi et al. demonstrated that endothelial endoglin is involved in inflammation and plays a role in leukocyte adhesion and transmigration *in vitro* and *in vivo*. In addition, cell adhesion molecules participated in this process with endoglin, suggesting that endoglin might be considered as a cell adhesion molecule (Rossi et al., 2013).

Both P-selectin and VCAM-1 represent crucial cell adhesion molecules that are the hallmarks of endothelial dysfunction and atherogenesis (Joseph-Silverstein and Silverstein, 1998). Their expression is increased by several stimuli, including cholesterol diet, as demonstrated in rabbits, mice and humans (Davies et al., 1993; Li et al., 1993; Nakashima et al., 1998). Both P-selectin and VCAM-1 are proatherogenic and proinflammatory markers strongly expressed in vascular endothelium in atherosclerosis prone arteries (Cybulsky et al., 2001; Ley and Huo, 2001).

Our previous papers showed endoglin expression exclusively in endothelium of aortic sinus in mice with advanced atherosclerosis (Rathouska et al., 2011; Strasky et al., 2011; Vecerova et al., 2012). However, a detailed immunohistochemical analysis of endoglin expression in aortic sinus and aorta during progression of atherosclerosis has not been reported so far. In addition, a possible expression of endoglin with pro-inflammatory P-selectin and VCAM-1 has not been demonstrated *in vivo* in aorta.

In this study, we set two goals. The first aim was the evaluation of endoglin expression in two different parts of aorta (heart aortic sinus and ascending aorta) in apoE-deficient mice fed either chow or an atherogenic diet (Western type diet). The second aim was the examination of a possible simultaneous expression of endoglin with P-selectin and VCAM-1, in order to evaluate a potential cooperation of endoglin with cell adhesion molecules in apoE-deficient mice during atherogenesis.

## Materials and methods

### *Animals*

Animal studies met the accepted criteria for human care and experimental use of laboratory animals. All protocols were approved by the Ethical Committee for the protection of animals against cruelty at Faculty of Pharmacy, Charles University in Prague and all experiments were carried out in accordance with Czech law No. 246/1992.

Ten-week–old female apoE-deficient mice on a C57BL/6J background (n=24) (Taconic, Denmark) were randomly subdivided into three groups.

All mice were fed with two different experimental diets with water *ad libitum* throughout the study. The Chow group of animals (n=8) was fed with chow diet for another two months. There were two Western type diet groups; in the first group (n=8), Western type diet containing 21% fat (11% saturated fat) and 0.15% cholesterol was fed for another two months, in the second group (n=8), the same diet was fed for another four months.

Each mouse, in all groups, lived in a separate cage obtaining 4 g of food (in specially prepared pellets) daily. The food consumption was monitored every day. No differences in food consumption were visible, either between animals of one experimental group or between experimental groups.

At the end of the treatment period, all animals were fasted overnight and euthanized. The aortas, attached to the top half of the heart, were removed and then immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Prague, Czech Republic), snap frozen in liquid nitrogen and stored at -80°C before immunohistochemical staining. Blood samples were taken from vena cava inferior into heparin-coated tubes and centrifuged at 9000 rpm for 15 min. Collected plasma samples were subsequently stored at -80°C before biochemical analysis.

### *Biochemical analysis*

Serum lipoprotein fractions were prepared using sodium chloride density gradient ultracentrifugation (Beckman TL 100, Palo Alto, CA, USA). The lipoprotein fractions were distinguished in the following density ranges: very low-density lipoprotein (VLDL <1.006 g/ml; LDL <1.063 g/ml; high-density lipoprotein (HDL) >1.063 g/ml. The total cholesterol and lipoprotein fraction concentration of cholesterol were measured enzymatically by conventional enzymatic diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, triglycerides at 540 nm, ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

### *Immunohistochemistry*

Aortic sinus and ascending aortas from three groups of mice (one chow diet group and two Western type diet groups) were taken for analysis. Sequential tissue sectioning started in the mouse heart until the aortic root containing semilunar valves together with the aorta appeared. From this point on, serial cross-sections (7 μm) were cut on a cryostat and placed on gelatin-coated slides. The systematic uniform random sampling was performed for fifty sections from each mouse. Each tenth slide was taken and five slides from each mouse were used for detection of each protein (endoglin, VCAM-1, P-selectin, macrophages). The slides were primarily incubated with 5% non-fat dry milk in phosphate buffered saline (PBS) solution for 30 minutes.

For the detection of endoglin, VCAM-1 and macrophages, the slides were thereafter incubated with primary antibodies for 1 hour at room temperature and were developed with anti-rat ImmPRESSTM (mouse adsorbed) polymerized reporter enzyme staining system (Vector Laboratories, USA) in the presence of 200 μg/ml normal mouse IgG (Dako, Denmark) afterwards. For the detection of P-selectin, the slides were first incubated with anti-avidin and anti-biotin solutions (Vector Laboratories, USA). Afterwards, they were incubated with primary antibody for 1 hour at room temperature and then developed with biotin-conjugated horse anti-goat Ig (dilution 1:400 in BSA) (Vector Laboratories, USA) in the presence of 200 μg/ml normal mouse IgG (Dako, Denmark). In the case of all four antibodies, the antibody reactivity was detected by means of diaminobenzidine tetrahydrochloride substrate (Dako, Denmark). The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: monoclonal rat anti-mouse endoglin / CD105 (dilution 1/50 in BSA) and monoclonal rat anti-mouse VCAM-1 / CD106 (dilution 1/100 in BSA), both purchased from BD Pharmingen™, USA. For the staining of macrophages, monoclonal rat anti-mouse macrophages/monocytes antibody (dilution 1/100 in BSA) from AbD Serotec, UK was used. For the staining of P-selectin, polyclonal goat antibody (dilution 1/50 in BSA) directed to P-selectin (Santa Cruz Biotechnology, USA) was used.

Photo documentation and image digitizing from the microscope were performed with the Olympus AX 70, with a digital firewire camera Pixelink PL-A642 (Vitana Corporation, Canada) with image analysis software NIS ver 3.1 (Laboratory Imaging, Prague, Czech Republic).

### *Plaque size evaluation*

Quantitative analysis of plaque size was evaluated by means of orcein-hematoxylin histological staining and stereological methods. Sequential tissue sectioning and systematic uniform random sampling was performed for fifty sections from each mouse as mentioned above. Each sixth slide was used for orcein-hematoxylin staining. Thus, eight sections from each mouse were used for the stereological estimation. Briefly, the staining was performed by incubation of the slides in orcein solution for 30 minutes and thereafter counterstained with hematoxylin for 30 seconds. Photo documentation and image digitizing from the microscope were performed as mentioned above. Stereological analysis was performed with PointGrid module of the ELLIPSE software (ViDiTo, Slovakia) as previously described (Nachtigal et al., 2004).

## Results

### *Biochemical analysis of serum lipid levels in mice*

Analysis of blood lipid spectrum in apoE-deficient mice (Fig. 1) revealed significantly higher levels of total cholesterol in mice fed Western type diet for two months (Apo west 2m) in comparison with mice fed chow diet (29.96±2.59 vs. 10.98±0.87, P<0.001). Similarly, comparison of total cholesterol levels between mice fed Western type diet for four months (Apo west 4m) and mice fed chow diet (Apo chow) revealed significantly higher levels in Apo west 4m group (25.60±0.10 vs. 10.98±0.87, P<0.001). There were no significant changes between Apo west 2m and Apo west 4m in total cholesterol levels.

VLDL levels were significantly higher in Apo west 2m group in comparison with Apo chow group (20.55±2.01 vs. 7.98±0.74, P<0.001). Similarly, VLDL levels were significantly higher in Apo west 4m group in comparison with Apo chow group (19.27±0.75 vs. 7.98±0.74, P<0.001). There were no significant changes

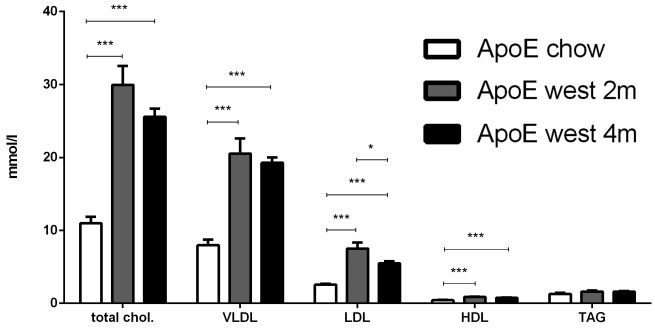


**Fig. 1.** Blood cholesterol levels in mice on chow diet and Western type diet for two and four months. Values are means ± SEM, n=8. ***P<0.001, *P<0.05.

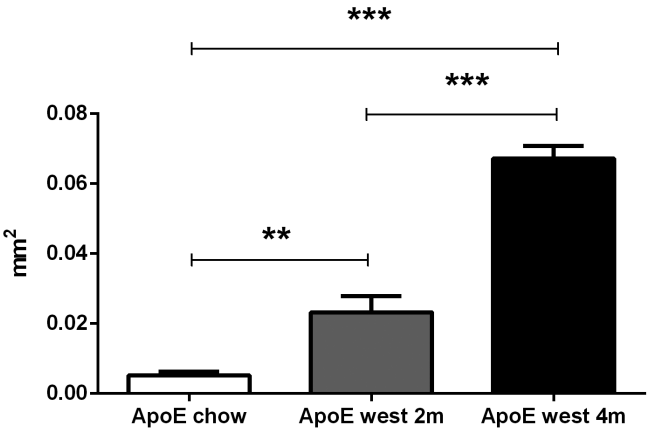


**Fig. 2.** Stereological analysis of lesion area size in aorta of mice on chow diet and Western type diet for two and four months. Lesion area size continually increased according to diet used and the period of feeding. Values are means ± SEM, n=8. ***P<0.001, **P<0.01.

between Apo west 2m and Apo west 4m in VLDL levels.

LDL levels were significantly higher in Apo west 2m group in comparison with Apo chow group (7.51±0.85 vs. 2.56±0.13, P<0.001). Similarly, LDL levels were significantly higher in Apo west 4m group in comparison with Apo chow group (5.48±0.30 vs. 2.56±0.13, P<0.001). There were also significantly higher levels of LDL in Apo west 2m mice in comparison with Apo west 4m mice (7.51±0.85 vs. 5.48±0.30, P<0.05).

HDL levels were significantly higher in Apo west 2m group in comparison with Apo chow group (0.87±0.07 vs. 0.44±0.04, P<0.001). Similarly, HDL levels were significantly higher in Apo west 4m group in comparison with Apo chow group (0.75±0.06 vs. 0.44±0.04, P<0.001). There were no significant changes between Apo west 2m and Apo west 4m in HDL levels. There were also no significant changes in triglyceride (TAG) levels between any compared groups (Fig. 1).

### *Atherosclerosis lesion size quantification*

The size of atherosclerotic lesions was assessed by stereological analysis of orcein-hematoxylin staining. Administration of Western type diet was associated with a larger plaque size in both Western type diet groups (Apo west 2m, Apo west 4m) in comparison with chow diet group (Apo chow). The plaque size was significantly larger in Apo west 2m group in comparison with Apo chow group (0.023±0.005 vs. 0.005±0.001 $mm^2$, P<0.01). The plaque size was also significantly larger in Apo west 4m group in comparison with Apo chow group (0.067±0.004 vs. 0.005±0.001 $mm^2$, P<0.001). The plaque size was even significantly larger in ApoE west 4m group in comparison with ApoE west 2m group (0.067±0.004 vs. 0.023±0.005 $mm^2$, P<0.001) (Fig. 2).

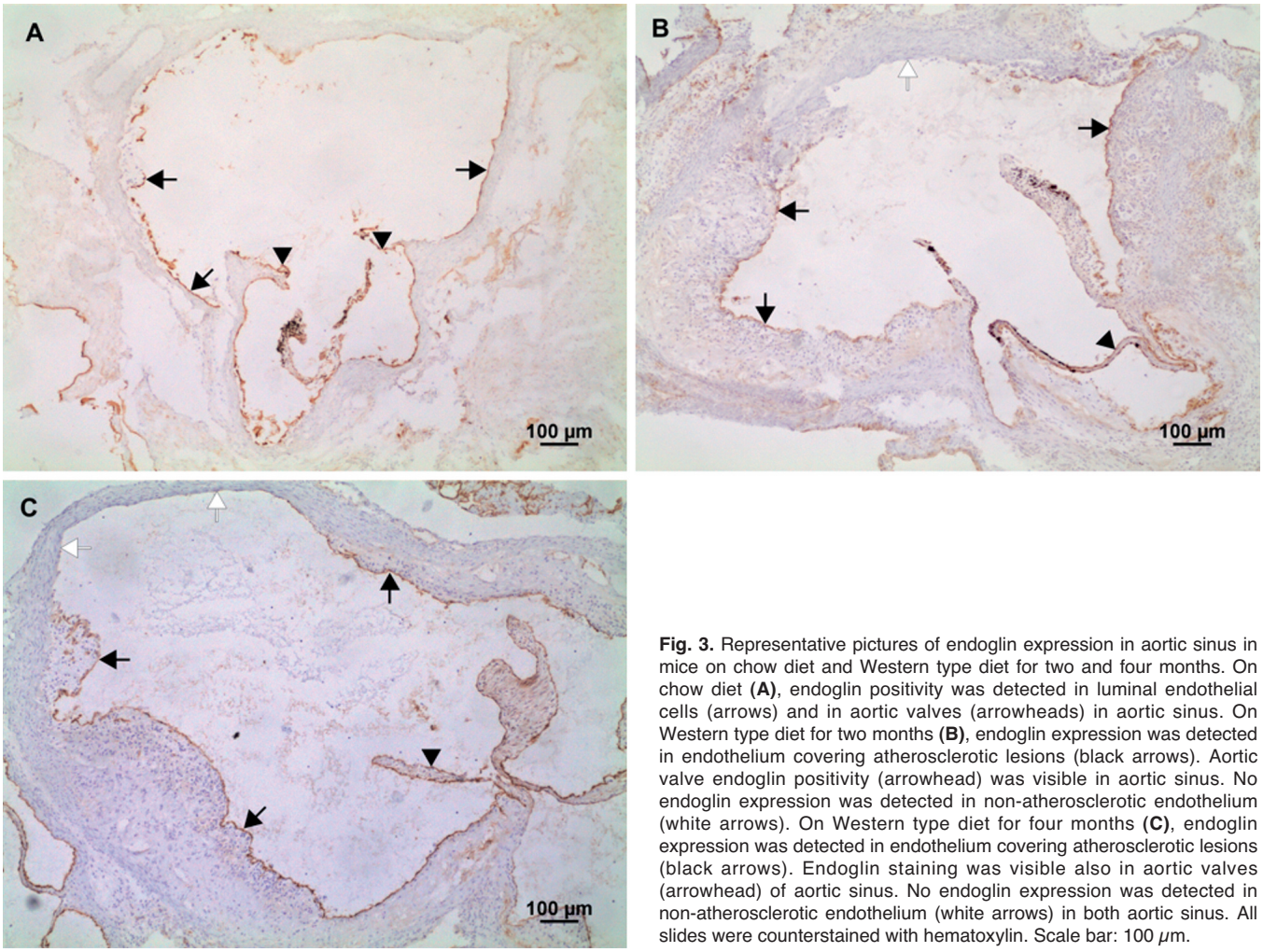


**Fig. 3.** Representative pictures of endoglin expression in aortic sinus in mice on chow diet and Western type diet for two and four months. On chow diet **(A)**, endoglin positivity was detected in luminal endothelial cells (arrows) and in aortic valves (arrowheads) in aortic sinus. On Western type diet for two months **(B)**, endoglin expression was detected in endothelium covering atherosclerotic lesions (black arrows). Aortic valve endoglin positivity (arrowhead) was visible in aortic sinus. No endoglin expression was detected in non-atherosclerotic endothelium (white arrows). On Western type diet for four months **(C)**, endoglin expression was detected in endothelium covering atherosclerotic lesions (black arrows). Endoglin staining was visible also in aortic valves (arrowhead) of aortic sinus. No endoglin expression was detected in non-atherosclerotic endothelium (white arrows) in both aortic sinus. All slides were counterstained with hematoxylin. Scale bar: 100 $\mu$m.

*Endoglin expression in mice on chow diet and on Western type diet for two and four months*

Immunohistochemical analysis of endoglin expression was performed in the aortic sinus inside the heart and in the ascending part of aorta in all studied animals.

In general, endoglin expression was detected only in endothelial cells in all studied animals. Endoglin positivity was visible in myocardial capillaries and in endocardial simple squamous epithelial cells (data not shown). Endoglin expression in aorta was detected only in luminal endothelial cells but not in the vessel media.

In mice on chow diet, endoglin expression covered almost the whole luminal area, including non-atherosclerotic endothelium and endothelium covering small atherosclerotic lesions in aortic sinus. Moreover, endoglin positivity was also detected in aortic valves of the aortic sinus (Fig. 3A). On the contrary, almost no endoglin expression was detected in aortic endothelium in ascending aorta, where no atherosclerotic lesions were present (Fig. 4A).

Atherosclerotic lesions were visible in both aortic sinus and ascending aorta in mice fed Western type diet for two and four months.

In mice fed Western type diet for two months, endoglin expression was detected almost exclusively in aortic endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium in aortic sinus (Fig. 3B). Moreover, endoglin expression was also detected in myocardial capillaries and aortic valves (Fig. 3B). Similarly, like in mice on chow diet, endoglin expression was weaker in aorta when compared to aortic sinus. In addition, endoglin was detected almost exclusively in endothelium covering atherosclerotic plaques but not in non-atherosclerotic endothelium in ascending aorta (Fig. 5A). In some parts of vessels, where no atherosclerotic lesions were visible, no

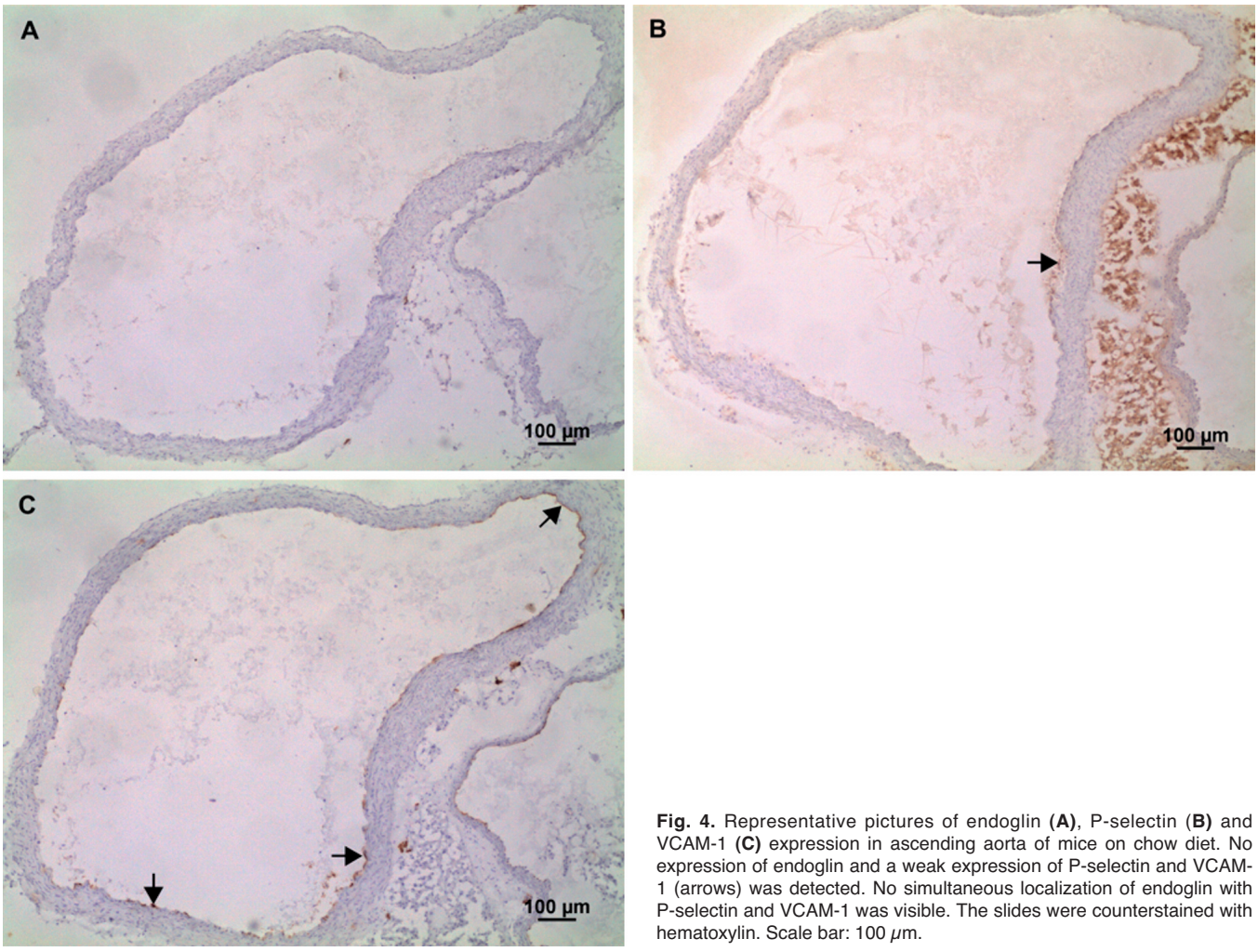


**Fig. 4.** Representative pictures of endoglin **(A)**, P-selectin (**B)** and VCAM-1 **(C)** expression in ascending aorta of mice on chow diet. No expression of endoglin and a weak expression of P-selectin and VCAM-1 (arrows) was detected. No simultaneous localization of endoglin with P-selectin and VCAM-1 was visible. The slides were counterstained with hematoxylin. Scale bar: 100 $\mu$m.

endoglin expression was detected (data not shown).

Similar staining patterns of endoglin expression were visible in mice on Western type diet for four months. Endoglin expression was detected almost exclusively in aortic endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium in aortic sinus (Fig. 3C). In addition, endoglin staining was visible in aortic valves of aortic sinus (Fig. 3C). Endoglin expression was also detected almost exclusively in aortic endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium in ascending aorta (Fig. 6A). In areas with no atherosclerotic lesions, no endoglin expression was visible (data not shown). Endoglin expression seemed to be weaker in aorta when compared to aortic sinus.

*Analysis of simultaneous endoglin expression with P-selectin and VCAM-1 in ascending aorta*

Since endoglin expression did not reflect the atherogenic process in aortic sinus, we decided to evaluate its expression with P-selectin and VCAM-1 only in ascending aorta, where changes of endoglin expression in the course of atherogenesis were detected. In general, P-selectin expression was detected only in endothelium, while VCAM-1 was also visible inside atherosclerotic lesions and in vessel media in ascending aorta.

A weak P-selectin and a stronger VCAM-1 expression was detected only in aortic endothelium in mice on chow diet. No endoglin expression in these vessels means no simultaneous localization of endoglin, P-selectin and VCAM-1 in mice on chow diet (Fig. 4A-C).

A strong endoglin endothelial staining and a substantially weaker P-selectin endothelial expression was detected in mice fed Western type diet for two months (Fig. 5A,B, respectively). VCAM-1 expression was visible inside the atherosclerotic lesions and in aortic media mostly underneath the atherosclerotic

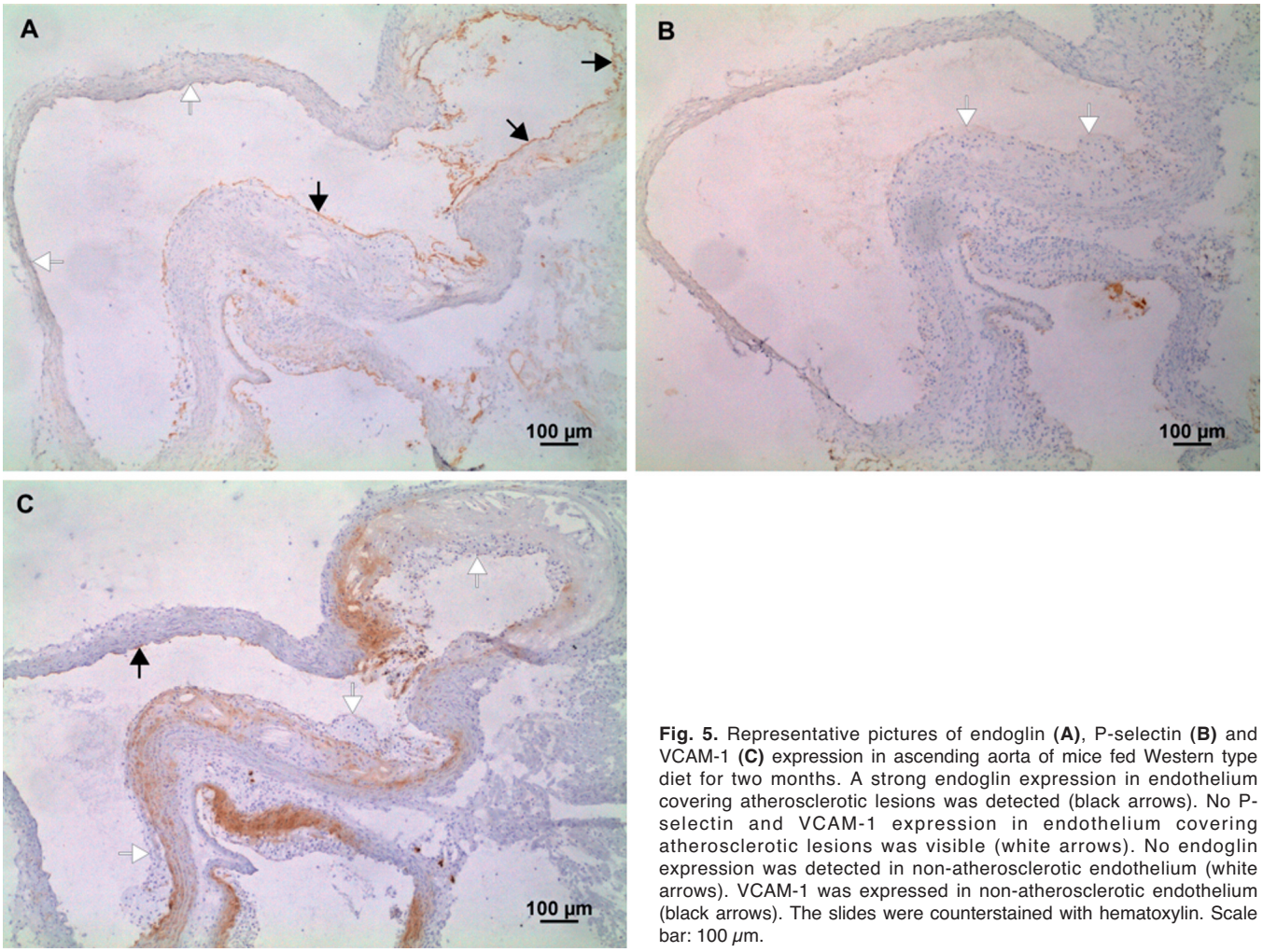


**Fig. 5.** Representative pictures of endoglin **(A)**, P-selectin **(B)** and VCAM-1 **(C)** expression in ascending aorta of mice fed Western type diet for two months. A strong endoglin expression in endothelium covering atherosclerotic lesions was detected (black arrows). No P-selectin and VCAM-1 expression in endothelium covering atherosclerotic lesions was visible (white arrows). No endoglin expression was detected in non-atherosclerotic endothelium (white arrows). VCAM-1 was expressed in non-atherosclerotic endothelium (black arrows). The slides were counterstained with hematoxylin. Scale bar: 100 $\mu$m.

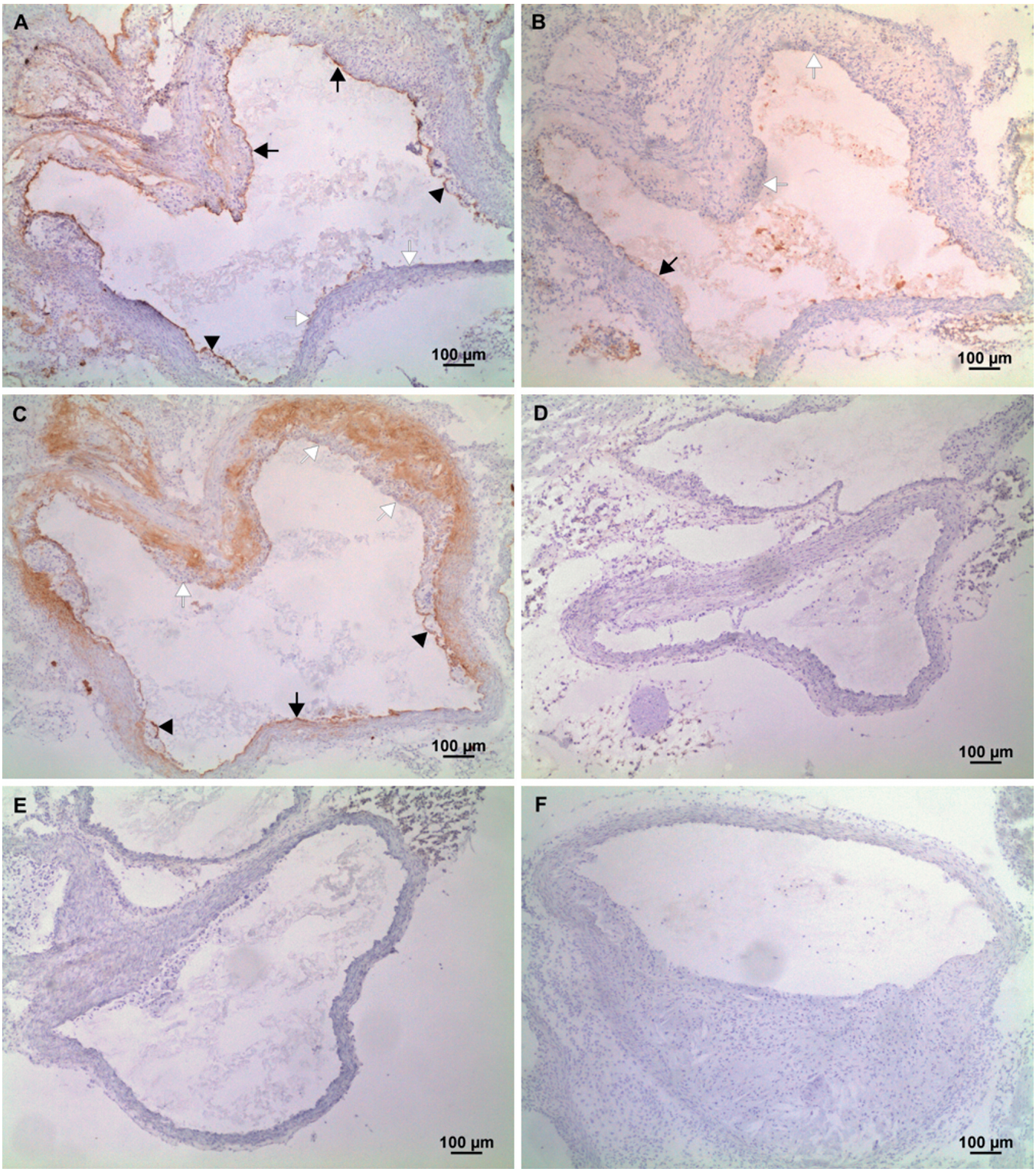


**Fig. 6.** Representative pictures of endoglin **(A)**, P-selectin **(B)** and VCAM-1 **(C)** expression in ascending aorta in mice fed Western type diet for four months. A strong endoglin expression in endothelium covering atherosclerotic lesions was detected (black arrows). No P-selectin and VCAM-1 expression in endothelium covering atherosclerotic lesions was visible (white arrows). No endoglin expression was detected in non-atherosclerotic endothelium (white arrows). Occasional VCAM-1 expression with endoglin was visible in small fatty streaks (black arrowheads). VCAM-1 and P-selectin were slightly expressed in non-atherosclerotic endothelium (black arrows). Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for endoglin **(D)**, P-selectin **(E)** and VCAM-1 **(F)**. The slides were counterstained with hematoxylin. Scale bar: 100 $\mu$m.

lesions. Moreover, VCAM-1 expression was detected in non-atherosclerotic endothelium and weakly in some vessels in endothelium covering atherosclerotic lesions (Fig. 5C). No substantial localization of endoglin with P-selectin and VCAM-1 was detected in mice fed Western type diet for two months (Fig. 5A-C).

Similar staining patterns of endoglin, P-selectin and VCAM-1 expression were demonstrated in mice aorta of mice fed Western type diet for four months (Fig. 6A-C). Endoglin expression was visible only in endothelium covering atherosclerotic plaques, P-selectin expression was hardly detected and VCAM-1 expression was visible inside the atherosclerotic lesions, in aortic media in non-atherosclerotic endothelium and in some vessels in endothelium covering small fatty streaks. A weak simultaneous expression of endoglin and VCAM-1 but not with P-selectin was visible only in some vessels in endothelium covering small fatty streaks (Fig. 6A-C).

### *Macrophages staining in aorta of apoE-deficient mice*

Immunohistochemical staining showed an accumulation of macrophages during the atherogenic process. No or a weak positivity for macrophages accumulated in aortic intima was visible in most of the vessels in mice on chow diet (Fig. 7A). On the other hand, the accumulation of macrophages in atherosclerotic lesions was visible in mice fed Western type diet for two and four months (Fig. 7B,C). Macrophage accumulation was stronger in mice fed Western type diet for four months when compared to mice fed the diet for two months (Fig. 7B,C).

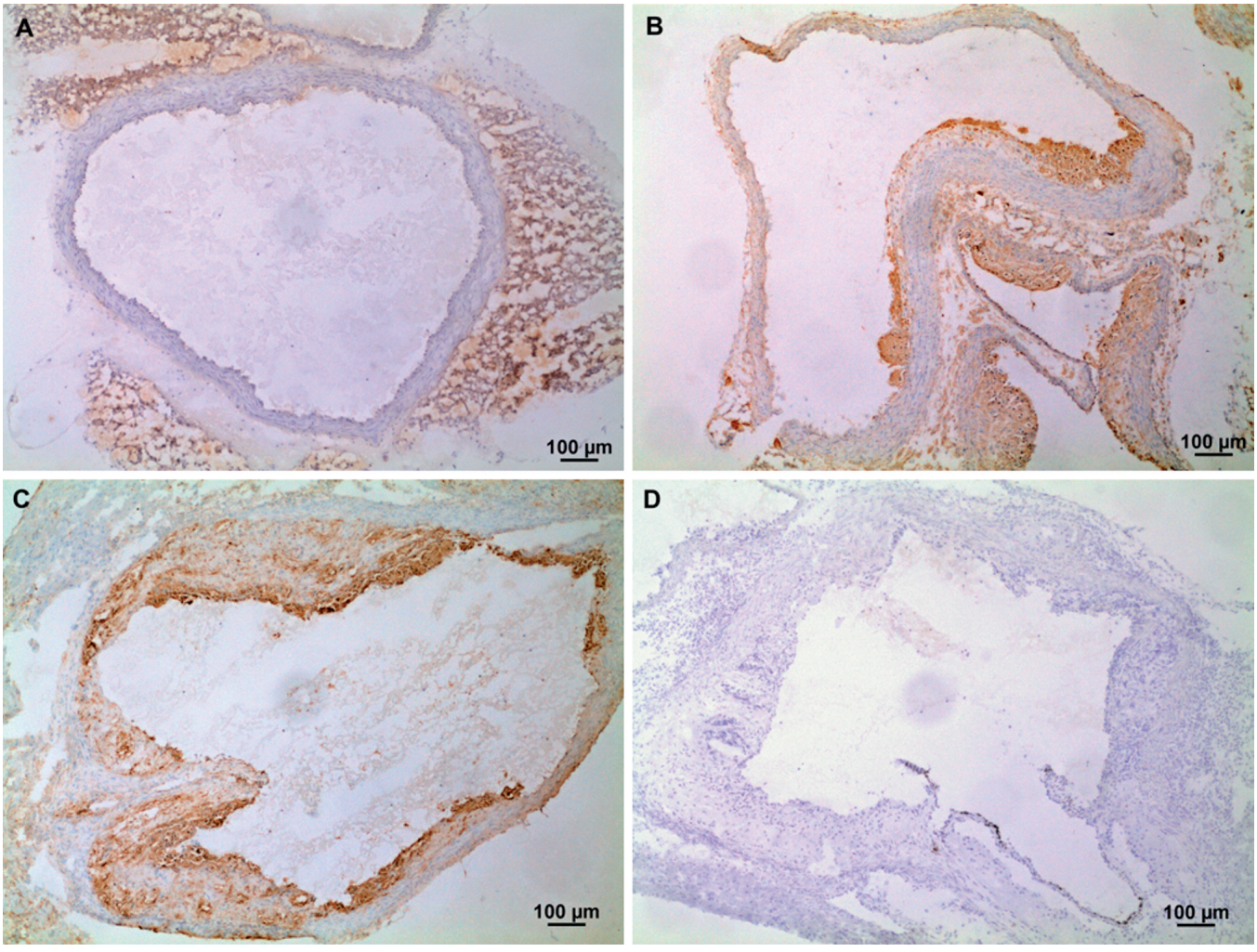


**Fig. 7.** Representative pictures of macrophage staining in mice on chow diet **(A)** and Western type diet for two **(B)** and four **(C)** months. Macrophage accumulation in intima was barely detectable in mice on chow diet but it was clearly visible in mice fed Western type diet for two and four months. Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for macrophages **(D)**. The slides were counterstained with hematoxylin. Scale bar: 100 *µ*m.

## Discussion

Endoglin expression in atherosclerosis has been studied in both humans and experimental animals. In general, endoglin expression was detected in cells that are important for atherogenesis and vascular homeostasis, including endothelial cells, monocyte/macrophages, as well as in vascular smooth muscle cells (Nachtigal et al., 2012). However, controversial data come from these studies with respect to endoglin localization in atherosclerotic vessels and its possible role in atherogenesis.

Therefore, the first aim of this study was to evaluate changes of endoglin localization in the part of aorta located inside the heart (aortic sinus) and in ascending aorta located outside the heart. Secondly, we wanted to evaluate a possible simultaneous expression of endoglin with cell adhesion molecules involved in atherogenesis (P-selectin, VCAM-1).

For that purpose we used apoE-deficient mice fed chow diet, Western type diet (two months) and Western type diet (four months) in order to see possible changes of endoglin expression during different phases of the atherogenic process. Indeed, a cholesterol diet significantly increased the levels of cholesterol, increased the atherosclerotic plaque size and resulted in macrophage accumulation in aortic plaques. These changes in the aorta allowed us to study endoglin expression changes with respect to development of atherosclerosis in aorta.

Most of the studies published in humans showed endoglin expression almost exclusively in endothelium of microvessels. Li et al. showed a weak or no expression of endoglin in normal arteries but a strong expression in atherosclerotic carotid arteries, predominantly in microvessels of the lipid core (Li et al., 2006). Additionally, endoglin expression was strong in neovessels within the carotid plaque, predominantly within vulnerable plaque shoulders, and within the lipid core (Luque et al., 2008). In another study, endoglin expression was strongest in advanced lesions in neovessels in the human carotid and coronary arteries when compared with early lesions (Luque et al., 2009). More recently, Li et al. showed that endoglin expression in coronary artery microvessels correlated with plaque hemorrhages (Li et al., 2012).

These studies suggested a possible participation of endoglin in plaque neoangiogenesis and in the regulation of intraplaque inflammation, because these plaque microvessels can serve as a gateway for the recruitment of inflammatory cells into atherosclerotic plaques (de Boer et al., 1999). On the contrary, another study also showed endoglin expression in carotid plaque neovessels, although these authors suggested that endoglin together with other markers might be important for the formation of neovessels less prone to leakage, rupture and hemorrhage (Slevin et al., 2010). Surprisingly, there was no information about endoglin expression in smooth muscle cells, macrophages or luminal endothelial cells in these studies.

On the other hand, some other papers showed different endoglin expression in non-atherosclerotic and atherosclerotic vessels. Conley et al. detected a weak expression of endoglin in non-atherosclerotic aortas and carotid arteries but a strong endoglin expression in advanced atherosclerotic plaques in smooth muscle cells, again with no positivity in luminal endothelial cells or macrophages (Conley et al., 2000). Piao et al. demonstrated a weak endoglin expression in normal arteries but a higher expression of endoglin in macrophages, smooth muscle cells and endothelial cells in early lesions when compared with advanced lesions (Piao and Tokunaga, 2006). These papers suggested that endoglin participates in vascular repair and possibly in atherogenesis.

Surprisingly, Bot et al. focused on the expression of endoglin in human carotid endarterectomy showing endoglin expression in intraplaque vessels (endothelial cells), macrophages and smooth muscle cells. Moreover, endoglin expression was higher in plaques containing higher levels of collagen and less thrombi in the plaque, suggesting that endoglin expression is related to a more stable plaque phenotype (Bot et al., 2009).

These discrepancies in human studies might be related to the fact that different vessels in different stages of atherosclerosis were studied and we cannot rule out the possibility of different reactions of various antibodies used in these studies.

It is of interest to mention that no human study focusing on the expression of endoglin during atherogenesis mentioned endoglin expression in aortic, carotid or coronary luminal endothelial cells and there has also been no data from experimental studies in mice concerning endoglin expression in these parts of the vascular tree so far.

We showed endoglin expression in endothelial cells in myocardial capillaries and luminal endothelial cells in aortic sinus and aorta of apoE-deficient mice. In addition, endoglin expression was also detected in aortic valves and simple squamous epithelium in endocardium in this study. This is in line with previously published papers showing that endoglin is not expressed by smooth muscle and macrophages in mice atherosclerosis (Nachtigal et al., 2009b; Rathouska et al., 2011; Strasky et al., 2011; Vecerova et al., 2012). On the other hand, no neovessels were visible in our mice. Endoglin expression in luminal endothelial cells was very strong inside the heart in aortic sinus. In fact, almost all luminal endothelial cells were stained for endoglin, regardless of the presence or stage of atherosclerotic process. Considering endoglin expression in aortic valves and endocardium, we might propose that endoglin expression in aortic sinus is not related to the progression of atherosclerosis/atherogenesis but might more likely be related to the role of endoglin in heart and valve development as demonstrated previously (Qu et al., 1998).

On the other hand, a different reaction was observed

in the aorta outside the heart. In general, endoglin positivity was weaker in this part of aorta when compared to aortic sinus in all studied groups. In addition, endoglin expression was almost absent in aortic endothelium in vessels or parts of vessels where no atherosclerotic lesions were found, irrespective of the diet. In other words, concerning the parts of aorta with no relation to heart and valve development, endoglin expression was detected only in endothelial cells on the surface of atherosclerotic lesions in all mouse groups. Our previous papers showed endoglin co-localization with eNOS in mice atherosclerosis (Nachtigal et al., 2009a,b; Vecerova et al., 2012). In addition, endoglin is able to affect expression and activity of eNOS (Jerkic et al., 2004; Toporsian et al., 2005; Cudmore et al., 2007). We might speculate that endoglin expressed only by plaque endothelium might be related to its endothelial protective role together with eNOS. On the other hand, Rossi et al. demonstrated recently that endoglin might be involved in leukocyte adhesion during transmigration both *in vitro* and *in vivo* and cooperates in this process with cell adhesion molecules (Rossi et al., 2013). Despite very convincing data from the paper, it is necessary to point out that endoglin role in leukocyte adhesion was demonstrated in venules in the study. Indeed, venules are critical vessels for inflammatory reaction in most organs (Scalia, 2013). However, homeostasis of arteries as vessels prone to the development of atherosclerosis is different when compared to venules or capillaries (different size, structure and hemodynamic conditions).

VCAM-1 and P-selectin are critical cell adhesion molecules participating in the development of endothelial dysfunction and atherogenesis (Ramos et al., 1999; Ley and Huo, 2001). Early P-selectin and VCAM-1 endothelial expression was demonstrated even before the formation of atherosclerotic lesions (Li et al., 1993), suggesting that they are markers of endothelial activation. Because of the fact that endoglin reflected the atherogenic process only in ascending aorta in this study, we focused on endoglin expression with P-selectin and VCAM-1 only in this part of the vessel. Weak P-selectin and VCAM-1 expressions were detected in non-atherosclerotic endothelium, although no simultaneous expression with endoglin was observed, suggesting that endoglin is not involved in early activation of endothelium in mice atherogenesis. In addition, no simultaneous expression of endoglin with P-selectin and only a weak occasional expression of VCAM-1 and endoglin was visible in endothelium covering atherosclerotic plaques. Based on the results of this immunohistochemical study focusing on mice aorta with different phases of atherogenesis, we might propose that endoglin is not expressed together with cell adhesion molecules that are critical for inflammation and atherogenesis. These results, of course, do not rule out a possibility that endoglin participates in the inflammation and leukocyte adhesion in other parts of the vascular tree or other organs. It must be pointed out that endoglin plays different roles in various organs and in various pathological conditions. Endoglin related activation and overexpression of eNOS might be related to the improvement of endothelial dysfunction (Jerkic et al., 2004) (antiatherogenic effect) or activation of angiogenesis (pro-atherogenic) (Duda et al., 2004; Li et al., 2012). In addition, endoglin related increase of collagen production might increase the stability of atherosclerotic plaques (Bot et al., 2009) or support a cardiac fibrosis (Kapur et al., 2012).

It must be stated that immunohistochemical analysis used in this study cannot answer the question about the proatherogenic or antiatherogenic role of endoglin in atherosclerosis; however, it brings another piece of the puzzle with respect to evaluation the role of endoglin in atherogenesis.

In summary, we showed that: 1) endoglin expression is detected in endothelial cells but not in macrophages or smooth muscle cells in aortic sinus and aorta in apoE-deficient mice, 2) endoglin expression varies during the atherogenic process in aorta but not in aortic sinus, 3) endoglin expression is detected only in endothelium covering atherosclerotic lesions but not in non-atherosclerotic endothelium regardless of the plaque size and 4) endoglin is not expressed together with P-selectin and VCAM-1 in aortic endothelium.

In conclusion, this study shows that endothelial expression of endoglin is related to the atherogenic process predominantly in aorta outside the heart. Moreover, endoglin is not localized with cell adhesion molecules involved in atherosclerosis, suggesting it might not participate in leukocyte accumulation in aorta of apoE-deficient mice during atherogenesis.

*Acknowledgements.* This work was supported by grant from the Grant Agency of Charles University in Prague number 300911/C, Charles University in Prague project SVV/2013/267003. The publication is co-financed by the European Social Fund and the state budget of the Czech Republic, project no. CZ.1.07/2.3.00/30.0061.

## References

Bot P.T., Hoefer I.E., Sluijter J.P., van Vliet P., Smits A.M., Lebrin F., Moll F., de Vries J.P., Doevendans P., Piek J.J., Pasterkamp G. and Goumans M.J. (2009). Increased expression of the transforming growth factor-beta signaling pathway, endoglin, and early growth response-1 in stable plaques. Stroke 40, 439-447.

Conley B.A., Smith J.D., Guerrero-Esteo M., Bernabeu C. and Vary C.P. (2000). Endoglin, a tgf-beta receptor-associated protein, is expressed by smooth muscle cells in human atherosclerotic plaques. Atherosclerosis 153, 323-335.

Cudmore M., Ahmad S., Al-Ani B., Fujisawa T., Coxall H., Chudasama K., Devey L.R., Wigmore S.J., Abbas A., Hewett P.W. and Ahmed A. (2007). Negative regulation of soluble flt-1 and soluble endoglin release by heme oxygenase-1. Circulation 115, 1789-1797.

Cybulsky M.I., Iiyama K., Li H., Zhu S., Chen M., Iiyama M., Davis V.,

Gutierrez-Ramos J.C., Connelly P.W. and Milstone D.S. (2001). A major role for vcam-1, but not icam-1, in early atherosclerosis. J. Clin. Invest. 107, 1255-1262.

Davies M.J., Gordon J.L., Gearing A.J., Pigott R., Woolf N., Katz D. and Kyriakopoulos A. (1993). The expression of the adhesion molecules icam-1, vcam-1, pecam, and e-selectin in human atherosclerosis. J. Pathol. 171, 223-229.

de Boer O.J., van der Wal A.C., Teeling P. and Becker A.E. (1999). Leucocyte recruitment in rupture prone regions of lipid-rich plaques: A prominent role for neovascularization? Cardiovasc. Res. 41, 443-449.

Duda D.G., Fukumura D. and Jain R.K. (2004). Role of enos in neovascularization: No for endothelial progenitor cells. Trends Mol. Med. 10, 143-145.

Jerkic M., Rivas-Elena J.V., Prieto M., Carron R., Sanz-Rodriguez F., Perez-Barriocanal F., Rodriguez-Barbero A., Bernabeu C. and Lopez-Novoa J.M. (2004). Endoglin regulates nitric oxide-dependent vasodilatation. FASEB J. 18, 609-611.

Joseph-Silverstein J. and Silverstein R.L. (1998). Cell adhesion molecules: An overview. Cancer Invest. 16, 176-182.

Kapur N.K., Wilson S., Yunis A.A., Qiao X., Mackey E., Paruchuri V., Baker C., Aronovitz M.J., Karumanchi S.A., Letarte M., Kass D.A., Mendelsohn M.E. and Karas R.H. (2012). Reduced endoglin activity limits cardiac fibrosis and improves survival in heart failure. Circulation 125, 2728-2738.

Levine R.J., Lam C., Qian C., Yu K.F., Maynard S.E., Sachs B.P., Sibai B.M., Epstein F.H., Romero R., Thadhani R. and Karumanchi S.A. (2006). Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N. Engl. J. Med. 355, 992-1005.

Ley K. and Huo Y. (2001). Vcam-1 is critical in atherosclerosis. J. Clin. Invest. 107, 1209-1210.

Li H., Cybulsky M.I., Gimbrone M.A. Jr and Libby P. (1993). An atherogenic diet rapidly induces vcam-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler. Thromb. 13, 197-204.

Li C., Mollahan P., Baguneid M.S., McMahon R.F., Kumar P., Walker M.G., Freemont A.J. and Kumar S. (2006). A comparative study of neovascularisation in atherosclerotic plaques using cd31, cd105 and tgf beta 1. Pathobiology 73, 192-197.

Li X., van der Meer J.J., van der Loos C.M., Ploegmakers H.J., de Boer O.J., de Winter R.J. and van der Wal A.C. (2012). Microvascular endoglin (cd105) expression correlates with tissue markers for atherosclerotic plaque vulnerability in an ageing population with multivessel coronary artery disease. Histopathology 61, 88-97.

Lopez-Novoa J.M. and Bernabeu C. (2010). The physiological role of endoglin in the cardiovascular system. Am. J. Physiol. Heart. Circ. Physiol. 299, H959-974.

Luque A., Turu M., Juan-Babot O., Cardona P., Font A., Carvajal A., Slevin M., Iborra E., Rubio F., Badimon L. and Krupinski J. (2008). Overexpression of hypoxia/inflammatory markers in atherosclerotic carotid plaques. Front. Biosci. 13, 6483-6490.

Luque A., Slevin M., Turu M.M., Juan-Babot O., Badimon L. and Krupinski J. (2009). Cd105 positive neovessels are prevalent in early stage carotid lesions, and correlate with the grade in more advanced carotid and coronary plaques. J. Angiogenes Res. 1, 6.

Nachtigal P., Semecky V., Kopecky M., Gojova A., Solichova D., Zdansky P. and Zadak Z. (2004). Application of stereological methods for the quantification of vcam-1 and icam-1 expression in early stages of rabbit atherogenesis. Pathol. Res. Pract. 200, 219-229.

Nachtigal P., Pospisilova N., Vecerova L., Micuda S., Brcakova E., Pospechova K. and Semecky V. (2009a). Atorvastatin increases endoglin, smad2, phosphorylated smad2/3 and enos expression in apoe/ldlr double knockout mice. J. Atheroscler. Thromb. 16, 265-274.

Nachtigal P., Vecerova L., Pospisilova N., Micuda S., Brcakova E., Navarro Hernandez E., Pospechova K. and Semecky V. (2009b). Endoglin co-expression with enos, smad2 and phosphorylated smad2/3 in normocholesterolemic and hypercholesterolemic mice: An immunohistochemical study. Histol. Histopathol. 24, 1499-1506.

Nachtigal P., Zemankova Vecerova L., Rathouska J. and Strasky Z. (2012). The role of endoglin in atherosclerosis. Atherosclerosis 224, 4-11.

Nakashima Y., Raines E.W., Plump A.S., Breslow J.L. and Ross R. (1998). Upregulation of vcam-1 and icam-1 at atherosclerosis-prone sites on the endothelium in the apoe-deficient mouse. Arterioscler. Thromb. Vasc. Biol. 18, 842-851.

Perez-Gomez E., Del Castillo G., Santibañez J.F., Lopez-Novoa J.M., Bernabeu C. and Quintanilla M. (2010). The role of the TGF-beta coreceptor endoglin in cancer. ScientificWorldJournal 10, 2367-2384.

Piao M. and Tokunaga O. (2006). Significant expression of endoglin (cd105), TGFbeta-1 and TGFbeta r-2 in the atherosclerotic aorta: An immunohistological study. J. Atheroscler. Thromb. 13, 82-89.

Qu R., Silver M.M. and Letarte M. (1998). Distribution of endoglin in early human development reveals high levels on endocardial cushion tissue mesenchyme during valve formation. Cell Tissue Res. 292, 333-343.

Ramos C.L., Huo Y., Jung U., Ghosh S., Manka D.R., Sarembock I.J. and Ley K. (1999). Direct demonstration of p-selectin- and vcam-1-dependent mononuclear cell rolling in early atherosclerotic lesions of apolipoprotein e-deficient mice. Circ. Res. 84, 1237-1244.

Rathouska J., Vecerova L., Strasky Z., Slanarova M., Brcakova E., Mullerova Z., Andrys C., Micuda S. and Nachtigal P. (2011). Endoglin as a possible marker of atorvastatin treatment benefit in atherosclerosis. Pharmacol. Res. 64, 53-59.

Rossi E., Sanz-Rodriguez F., Eleno N., Duwell A., Blanco F.J., Langa C., Botella L.M., Cabanas C., Lopez-Novoa J.M. and Bernabeu C. (2013). Endothelial endoglin is involved in inflammation: Role in leukocyte adhesion and transmigration. Blood 121, 403-415.

Scalia R. (2013). The microcirculation in adipose tissue inflammation. Rev. Endocr. Metabolic Dis. 14, 69-76.

Slevin M., Turu M.M., Rovira N., Luque A., Baldellou M., Krupinski J. and Badimon L. (2010). Identification of a 'snapshot' of co-expressed angiogenic markers in laser-dissected vessels from unstable carotid plaques with targeted arrays. J. Vasc. Res. 47, 323-335.

Strasky Z., Vecerova L., Rathouska J., Slanarova M., Brcakova E., Kudlackova Z., Andrys C., Micuda S. and Nachtigal P. (2011). Cholesterol effects on endoglin and its downstream pathways in apoe/ldlr double knockout mice. Circ. J. 75, 1747-1755.

ten Dijke P., Goumans M.J. and Pardali E. (2008). Endoglin in angiogenesis and vascular diseases. Angiogenesis 11, 79-89.

Toporsian M., Gros R., Kabir M.G., Vera S., Govindaraju K., Eidelman D.H., Husain M. and Letarte M. (2005). A role for endoglin in coupling enos activity and regulating vascular tone revealed in

hereditary hemorrhagic telangiectasia. Circ. Res. 96, 684-692.
Vecerova L., Strasky Z., Rathouska J., Slanarova M., Brcakova E., Micuda S. and Nachtigal P. (2012). Activation of TGF-beta receptors and smad proteins by atorvastatin is related to reduced atherogenesis in apoe/ldlr double knockout mice. J. Atheroscler. Thromb. 19, 115-126.

Accepted September 11, 2015