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Endoglin co-expression with eNOS, SMAD2 and phosphorylated SMAD2/3 in normocholesterolemic and hypercholesterolemic mice: an immunohistochemical study

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Summary. Endoglin, a homodimeric transmembrane glycoprotein, is a part of the transforming growth factor-ß (TGF-ß) receptor cascade. It has been demonstrated that endoglin can affect TGF-ß signaling and eNOS expression by affecting SMAD proteins *in vitro*. We planned to go one step forward and evaluate whether endoglin is co-expressed with SMAD2, phosphorylated SMAD2/3 protein and eNOS in endothelium of normocholesterolemic C57BL/6J mice, and in advanced atherosclerotic lesions in hypercholesterolemic apoE/LDLr-deficient mice by means of fluorescence immunohistochemistry.

Female C57BL/6J mice were fed with a chow diet (standard laboratory diet) for 12 weeks after weaning (at the age of 4 weeks). Two-month-old female apoE/LDLr-deficient mice were fed the western type diet (atherogenic diet) containing 21% fat (11% saturated fat) and 0.15% cholesterol for 2 months. Immunohistochemical analysis of endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS expression was performed in mice aortic sinus.

Immunohistochemical analysis showed the expression of endoglin in intact endothelium in both C57BL/6J and apoE/LDLr-deficient mice and in endothelium covering the atherosclerotic lesion in apoE/LDLr-deficient mice. Fluorescence immunohistochemistry revealed co-expression of endoglin with SMAD2, phosphorylated SMAD2/3 and eNOS in intact aortic endothelium in C57BL/6J mice. Moreover, strong co-localization of endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS was also detected in endothelium covering atherosclerotic lesions in apoE/LDLr-deficient mice.

In conclusion, we suggest that endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS may be important in vessel endothelium homeostasis underlying their role in atherogenesis.

Key words: Endoglin, SMAD2, Phosphorylated SMAD2/3, eNOS, Immunohistochemistry, C57BL/6J mice, apoE/LDLr-deficient mice

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Introduction

Endoglin (CD 105) is a 190-kDa homodimeric transmembrane glycoprotein composed of 95-kDa disulfide-linked subunits (Guerrero-Esteo et al., 1999). The major source of CD 105 is vascular endothelial cells. Other cell types, including vascular smooth muscle cells (Adam et al., 1998), fibroblasts (St-Jacques et al., 1994), macrophages (Lastres et al., 1992), leukemic cells of pre-B and myelomonocytic origin (Kay et al., 2002), and erythroid precursors (Buhring et al., 1991) express CD105 to a lesser extent. Endoglin is highly expressed in endothelial cells of tissues undergoing angiogenesis, such as healing wounds, infarcts and in a wide range of tumors (Duff et al., 2003). We previously demonstrated that endoglin is expressed by aortic vessel endothelium in normo- and hypercholesterolemic mice (Nachtigal et al., 2006, 2007). Endoglin expression was also detected in human and porcine atherosclerotic lesions (Conley et al., 2000; Behr-Roussel et al., 2000; Ma et al., 2000; Piao and Tokunaga, 2006). Mutations in the gene encoding endoglin have been linked to the human disease hereditary hemorrhagic telangiectasia 1 (HHT1), an autosomal dominant inherited vascular disorder (Behr-Roussel et al., 2000).

Endoglin is a part of the transforming growth factor-

ß (TGF-ß) receptor cascade also known as a type III TGF-ß receptor. Endoglin forms complexes with heteromeric complexes of type I and type II serine/threonine kinase receptors (TßRI and TßRII), respectively, and has been postulated to affect TGF-ß1 signaling (Conley et al., 2000). Activation of TGF-ß signaling results in activation and translocation of the SMAD family of proteins to the nucleus to participate in regulating gene expression (Lebrin et al., 2005).

Eight Smad proteins are encoded in the human and mouse genomes. Only five of the mammalian Smads (Smad1, Smad2, Smad3, Smad5, and Smad8) act as substrates for the TGFß family of receptors; these are commonly referred to as receptor-regulated Smads, or RSmads (Massague, 1998). It has been demonstrated that endoglin may functionally interact with the endothelial-specific type I TGF-ß receptor ALK-1 to activate Smad1/5 and promote endothelial cell proliferation and migration. In contrast, endoglin interferes with ALK-5 signaling through Smad2/3, which normally inhibits endothelial cell proliferation and migration (Lebrin et al., 2005).

The endothelium plays a dual role in the regulation of vasomotor tone. It produces and releases both relaxing and constricting factors. The main vasorelaxing factor produced by endothelial cells (EC) is nitric oxide (NO) (Vanhoutte, 1997). NO possesses several important biological effects, including the inhibition of cell adhesion molecule expression and thus leukocyte adhesion to endothelium, inhibition of platelet aggregation and activation and inhibition of smooth muscle proliferation (Sessa, 2004). NO synthesis by endothelium is maintained by endothelial nitric oxide synthase (eNOS) which is constitutively expressed but also affected by different stimuli, including hypoxia, shear stress and LDL. It has been demonstrated that alteration of eNOS expression is related to the development and progression of atherosclerosis (Mungrue et al., 2003).

Recently it has been demonstrated that endoglin expression correlates with eNOS expression and NO-dependent vasodilatation (Jerkic et al., 2004), and that endoglin increases eNOS expression by modulating SMAD2 protein levels in endothelial cells *in vitro* (Santibanez et al., 2007). However, there is no available study showing that proteins of this proposed pathway are at least co expressed *in vivo* as well. Thus, in this study we wanted to evaluate whether endoglin is co-expressed with SMAD2, phosphorylated SMAD2/3 and eNOS in aortic endothelium of normocholesterolemic C57BL/6J mice and in advanced atherosclerotic lesions in hypercholesterolemic apoE/LDLr-deficient mice by means of immunohistochemistry.

Material and methods

Animals

Female C57BL/6J mice (n=8) (Taconic Europe, Lille Skensved, Denmark) were fed with a chow diet (standard laboratory diet) for 12 weeks after weaning (at the age of 4 weeks). Two-month-old female apoE/LDLr-deficient mice on a C57BL/6J background (n=8) (Taconic Europe, Lille Skensved, Denmark) were fed the western type diet (atherogenic diet) containing 21% fat (11% saturated fat) and 0.15% cholesterol by weight for 2 months. Each mouse in both 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. Blood samples were collected via cardiac puncture at the time of death. 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.

Immunohistochemistry

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. Sections were air-dried and then slides were fixed for 20 minutes in acetone at -20°C. For the detection of endoglin expression slides were rinsed in PBS (pH 7.4) and then incubated with anti avidin and anti biotin solutions (Vector Laboratories, USA). After blocking of nonspecific binding sites with 10% normal goat serum (Sigma-Aldrich Chemie, Germany) in PBS solution (pH 7.4) for 30 min, slides were incubated with primary antibodies for 1 hour at room temperature. After a PBS rinse, the slides were developed with biotin-conjugated goat anti-rat Ig (diluted 1/400 in BSA) (BD Pharmingen™, California, USA) in the presence of 200 µg/ml normal mouse IgG (Dako, Denmark). Antibody reactivity was detected using HRP (Horseradish peroxidase)-conjugated biotin-avidin complexes (Vector Laboratories, USA) and developed with diaminobenzidine tetrahydrochloride substrate (Dako, Denmark).

For the double fluorescence staining goat anti-rat secondary antibody marked with green fluorochrome (CY2) was used (diluted 1/100 in BSA) to detect endoglin. Goat anti-rabbit secondary antibody marked with red fluorochrome (CY3) was used (diluted 1/100 in BSA) for the detection of SMAD2, phosphorylated SMAD2/3 and eNOS. Both secondary antibodies were purchased from Jackson Immunoresearch (Suffolk, UK). The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies included the following: monoclonal antibody Rat Anti-Mouse Endoglin CD 105 (diluted 1:50) purchased from BD Pharmingen (California, USA), Rabbit polyclonal antibodies to

phosphorylated SMAD2/3 (diluted 1:100) and eNOS (diluted 1:100), obtained from SantaCruz Biotechnology, Inc. (California, USA) and Rabbit polyclonal antibody directed to SMAD2 (diluted 1:30), obtained from Abcam (Cambridge, UK).

Photo documentation and image digitizing from the microscope were performed with the Olympus AX 70 light and fluorescence microscope, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) and with VDS Vosskuehler CD-1300QB monochromatic camera for the fluorescence, with image analysis software NIS (Laboratory Imaging, Czech Republic).

Results

Immunohistochemical staining of endoglin in C57BL/6J mice and apoE/LDLr-deficient mice

Immunohistochemical staining was performed in the aortic sinus in all mice, which is the reference place for the study of mouse atherogenesis. The staining patterns of endoglin expression in both normocholesterolemic C57BL/6J and hypercholesterolemic apoE/LDLr-deficient mice were similar. The expression of endoglin was visible in the intact endothelium of both C57BL/6J and apoE/LDLr-deficient mice and in endothelium covering the atherosclerotic lesion in apoE/LDLr-deficient mice (Fig. 1A-D). Furthermore, strong staining was detected in the capillaries of surrounding myocardium and in aortic valves in both groups of mice (Fig. 1A-D). In some vessels of apoE/LDLr-deficient mice, weak endoglin staining was visible in the atherosclerotic intima, suggesting additional expression of endoglin by other intimal cells (data not shown).

Colocalization study of endoglin with SMAD2 phosphorylated SMAD2/3 and eNOS in C57BL/6J mice and apoE/LDLr-deficient mice

Double fluorescence staining of endoglin with SMAD2, phosphorylated SMAD2/3 and eNOS was also performed in aortic sinus in both C57BL/6J mice and apoE/LDLr-deficient mice. The expression of SMAD2 was detected by anti SMAD2 antibody, which should detect the inactivated (non-phosphorylated) form of SMAD2 in cells. The expression of phosphorylated SMAD2/3 was detected by anti SMAD2/3 antibody, which should detect the activated (phosphorylated) form of SMAD2/3.

The results revealed co-expression of endoglin with SMAD2 (Fig. 2A) and phosphorylated SMAD2/3 (Fig. 3A) in C57BL/6J mice in intact aorta endothelium. Strong co-localization of endoglin, SMAD2 (Fig. 2B) and phosphorylated SMAD2/3 (Fig. 3B) was also detected in endothelium covering atherosclerotic lesions in apoE/LDLr-deficient mice (Fig. 2B). Despite the fact that weak SMAD2 and strong phosphorylated SMAD2/3 expression was also found in atherosclerotic lesions, no co-expression of endoglin with these SMAD proteins was detected in the atherosclerotic lesion area.

Similar results were detected in the staining of endoglin with eNOS. Co-localization of both markers was visible in both C57BL/6J and apoE/LDLr-deficient mice in aortic endothelium only (Fig. 4A,B). No expression of eNOS was detected in the atherosclerotic lesion area. No co-expression of endoglin or eNOS was detected in surrounding myocardium and/or in the atherosclerotic lesion (data not shown).

Discussion

Endoglin (or CD105) is a homodimeric membrane glycoprotein that, in association with TGF-ß receptors, binds TGF-ß1 and - ß3 isoforms in human endothelial cells (Zhang et al., 1996). The role of endoglin in gene mutation was largely studied in hereditary hemorrhagic telangiectasia (Llorca et al., 2007). In addition, increased endoglin expression is observed in ECs of microvessels from pathological skin lesions and in the neovessels of tumors, suggesting a role for endoglin during EC proliferation (Letamendia et al., 1998).

The role of endoglin in atherogenesis has also been studied recently. Endoglin was expressed at low levels in normal porcine and human coronary arteries and overexpressed in diseased arteries, not only in ECs and fibroblasts, but also transiently in SMCs and macrophages (Conley et al., 2000; Behr-Roussel et al., 2000; Ma et al., 2000; Piao and Tokunaga, 2006). On the contrary, in this study we found the expression of endoglin predominantly in intact endothelium of both C57BL/6J and apoE/LDLr-deficient mice, and also in endothelium covering the atherosclerotic lesion in apoE/LDLr-deficient mice. Strong staining was also detected in the capillaries of surrounding myocardium and in aortic valves in both groups of mice. These results are consistent with our previous studies on non-atherosclerotic vessels in normo- and hyper-cholesterolemic mice (Nachtigal et al., 2006, 2007), suggesting that endoglin is expressed predominantly by vessel endothelium in mice.

Endothelial nitric oxide synthase (eNOS or NOS III) is a critical regulator of cardiovascular homeostasis, vascular remodeling, and angiogenesis, whose dysregulation leads to different types of vascular pathology, including atherosclerosis (Mungrue et al., 2003; Toborek and Kaiser, 1999). It has been shown that endoglin regulates nitric oxide-dependent vasodilatation, as well as eNOS expression and activity (Jerkic et al., 2004; Toporsian et al., 2005).

Moreover, SMAD proteins have been recognized as basic components of intracellular-signaling pathways of the TGF-ß family also affected by endoglin (Bea et al., 2003). The receptor-activated R-SMADs, such as SMAD2 and SMAD3, form heteromeric complexes with Co-SMAD (SMAD4). Such complexes subsequently translocate to the nucleus to regulate gene transcription (Massague et al., 2005).

It has also been demonstrated that endoglin increased eNOS expression via enhanced SMAD2

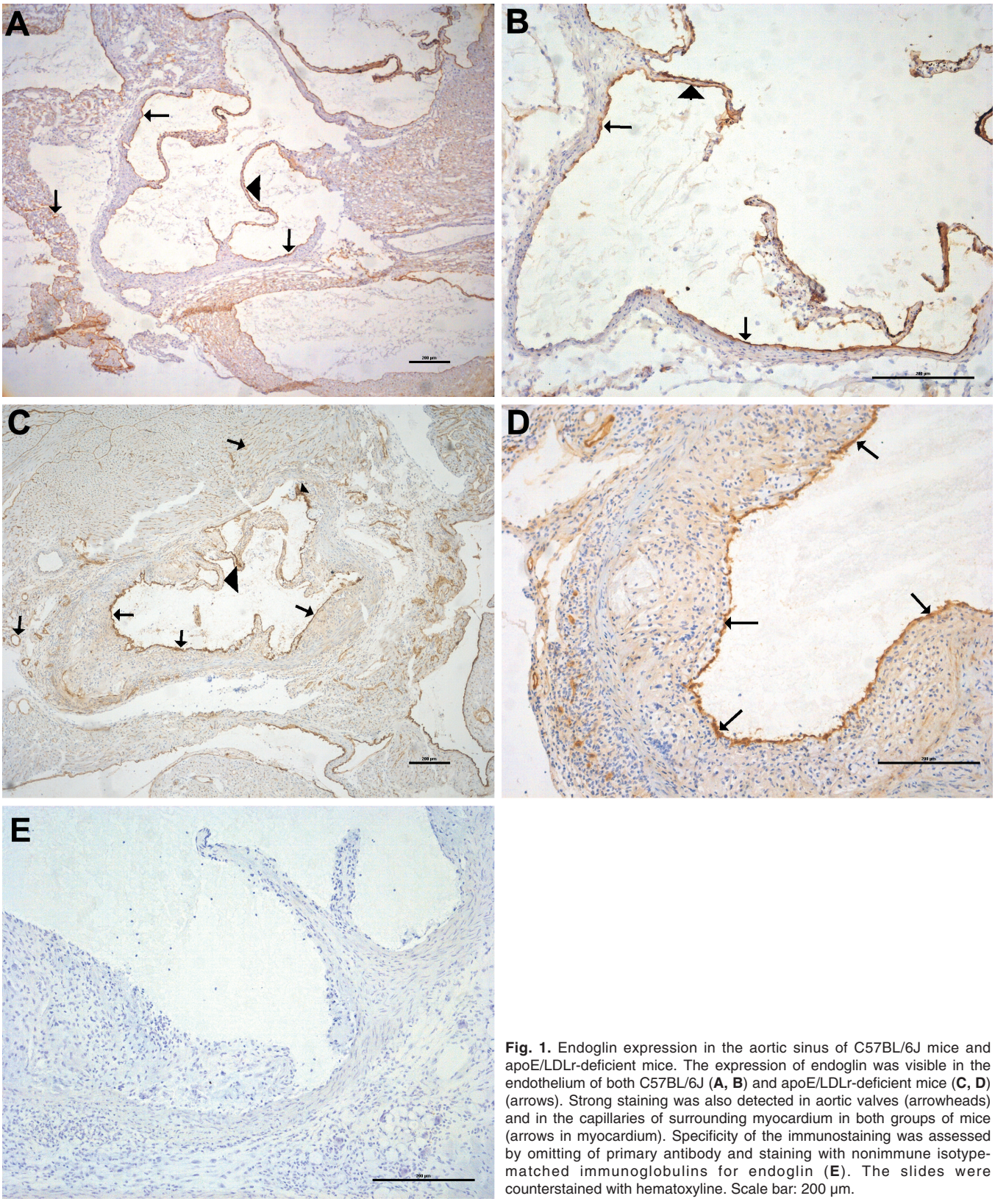

Fig. 1. Endoglin expression in the aortic sinus of C57BL/6J mice and apoE/LDLr-deficient mice. The expression of endoglin was visible in the endothelium of both C57BL/6J (**A, B**) and apoE/LDLr-deficient mice (**C, D**) (arrows). Strong staining was also detected in aortic valves (arrowheads) and in the capillaries of surrounding myocardium in both groups of mice (arrows in myocardium). Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for endoglin (**E**). The slides were counterstained with hematoxyline. Scale bar: 200 μm.

protein levels in endothelial cells *in vitro* (Santibanez et al., 2007).

The questions that we asked were: Is this pathway applicable even *in vivo?* Are those proteins at least co-expressed *in vivo*? To answer these questions we used two mouse models of atherosclerosis. We tested whether endoglin is co-expressed with SMAD2, activated (phosphorylated) SMAD2/3 and eNOS *in vivo* in both normocholesterolemic C57BL/6J mice and hypercholesterolemic, apoE/LDLr-deficient mice. Our results for the first time demonstrate the co-localization of endoglin and eNOS, as well as endoglin and both forms of SMAD2 proteins *in vivo*. The co-localization was visible in the intact aortic vessel endothelium of both C57BL/6J mice and apoE/LDLr-deficient mice, and in endothelium covering the atherosclerotic plaque of apoE/LDLr-deficient mice, suggesting their role in atherogenesis. The expression of SMAD2 was detected by anti SMAD2 antibody, which should detect the inactivated (non-phosphorylated) form of SMAD2 in cells. The expression of phosphorylated SMAD2/3 was detected by anti SMAD2/3 antibody, which should detect the activated (phosphorylated) form of SMAD2/3. Unfortunately, we could not use specific phosphorylated anti SMAD2 antibody due to technical problems with the detection of positivity, despite the fact that we tested anti phosphorylated SMAD2 antibodies from 2 different companies. However, we must emphasize that phosphorylated anti SMAD2/3 antibody should show positivity for both phospho-SMAD2 and phospho-SMAD3, according to the manufacturer's datasheet. Moreover, it has been demonstrated that SMAD3 is not expressed in endothelium, in contrast to SMAD2 (Feinberg and Jain, 2005). Therefore we can propose that phospho-SMAD2 is responsible for the endothelial staining and colocalization with endoglin in our study.

The results of this study are, however, partially opposite to the latest study showing that SMAD2 was

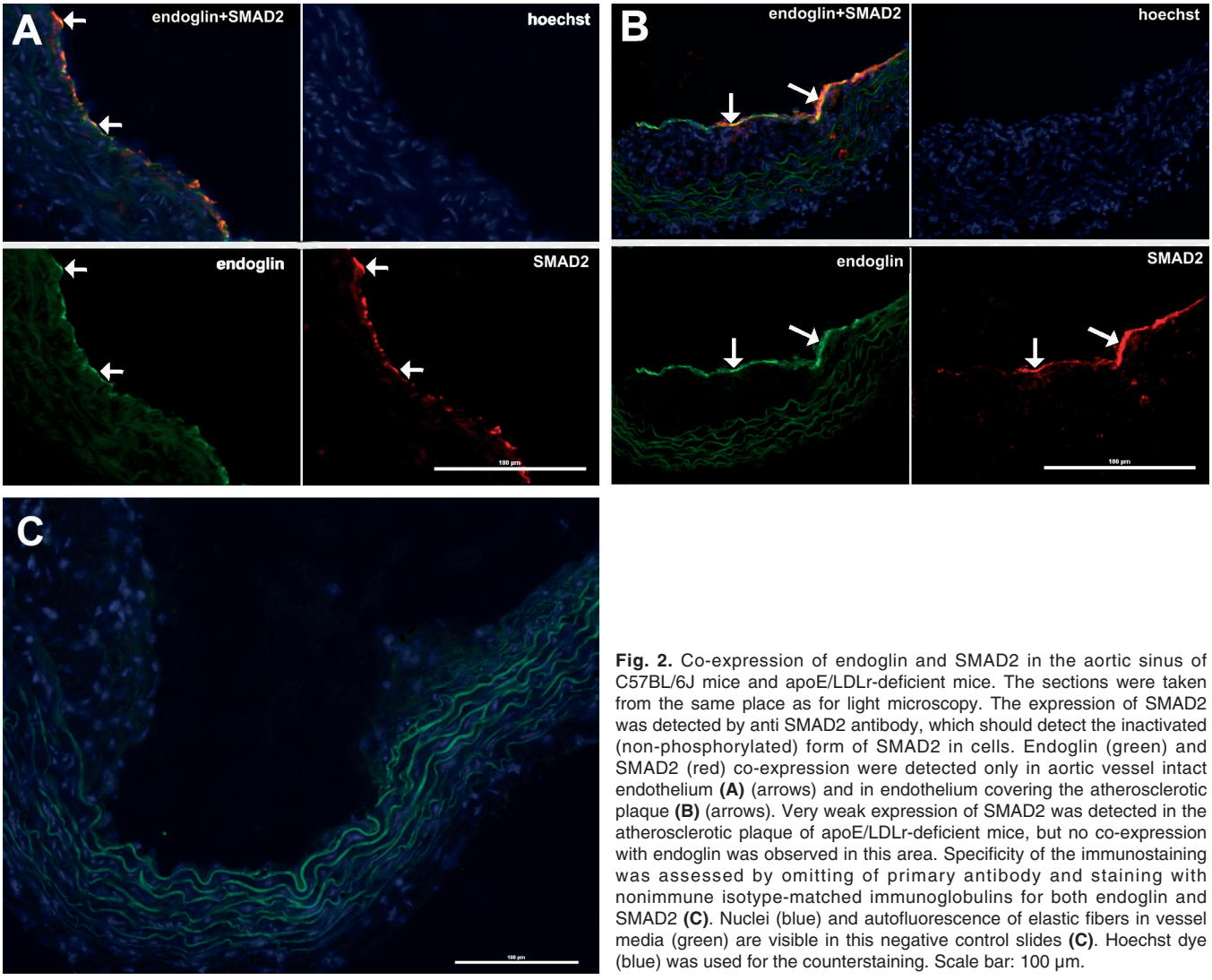

Fig. 2. Co-expression of endoglin and SMAD2 in the aortic sinus of C57BL/6J mice and apoE/LDLr-deficient mice. The sections were taken from the same place as for light microscopy. The expression of SMAD2 was detected by anti SMAD2 antibody, which should detect the inactivated (non-phosphorylated) form of SMAD2 in cells. Endoglin (green) and SMAD2 (red) co-expression were detected only in aortic vessel intact endothelium **(A)** (arrows) and in endothelium covering the atherosclerotic plaque **(B)** (arrows). Very weak expression of SMAD2 was detected in the atherosclerotic plaque of apoE/LDLr-deficient mice, but no co-expression with endoglin was observed in this area. Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for both endoglin and SMAD2 **(C)**. Nuclei (blue) and autofluorescence of elastic fibers in vessel media (green) are visible in this negative control slides **(C)**. Hoechst dye (blue) was used for the counterstaining. Scale bar: 100 μm.

expressed in normal intact arteries but not in atherosclerotic coronary vessels in apoE-deficient mice (Chen et al., 2007). This discrepancy might reflect the fact that we analyzed the expression of SMAD2 in aortic sinus but not in coronary arteries.

Endoglin forms complexes with TßRI and TßRII, and has been postulated to facilitate the binding of TGF-ß1 to these signaling receptors, suggesting that endoglin affects TGF-ß signaling events (Yamashita et al., 1994). Nevertheless, increasing evidence also indicates that endoglin may have functions independent of TGF-ß1. For example, only about 1% of the endoglin molecules on endothelial cells bind TGF-ß, suggesting that endoglin has another, undefined, physiological ligand (Conley et al., 2000). However, it still remains to be elucidated whether it is endoglin alone or endoglin together with TGF-ß that is able to regulate eNOS expression via the SMAD2 dependent pathway.

Previous papers demonstrated increased endoglin expression in atherosclerotic vessels when compared with normal vessels in both animals and humans (Conley et al., 2000; Behr-Roussel et al., 2000; Piao and Tokunaga, 2006). It was suggested, therefore, that endoglin might be a proatherogenic marker participating in the development and progression of atherosclerosis (Behr-Roussel et al., 2000; Conley et al., 2000; Ma et al., 2000; Piao and Tokunaga, 2006). However, the precise role of endoglin in atherogenesis remains unclear.

On the contrary, the increase of eNOS and SMAD2 protein expression by endoglin described recently (Santibanez et al., 2007) was also previously related to antiatherosclerotic effects in vessels (Laufs et al., 1998; Blair et al., 1999; Mallat and Tedgui, 2002). Very recently, SMAD2 levels were correlated with cholesterol levels and statin treatment *in vitro* and *in vivo* (Chen et al., 2008). It would be of interest to perform a co-localization *in vitro* study of endoglin, SMAD proteins and eNOS in endothelial cells under various conditions

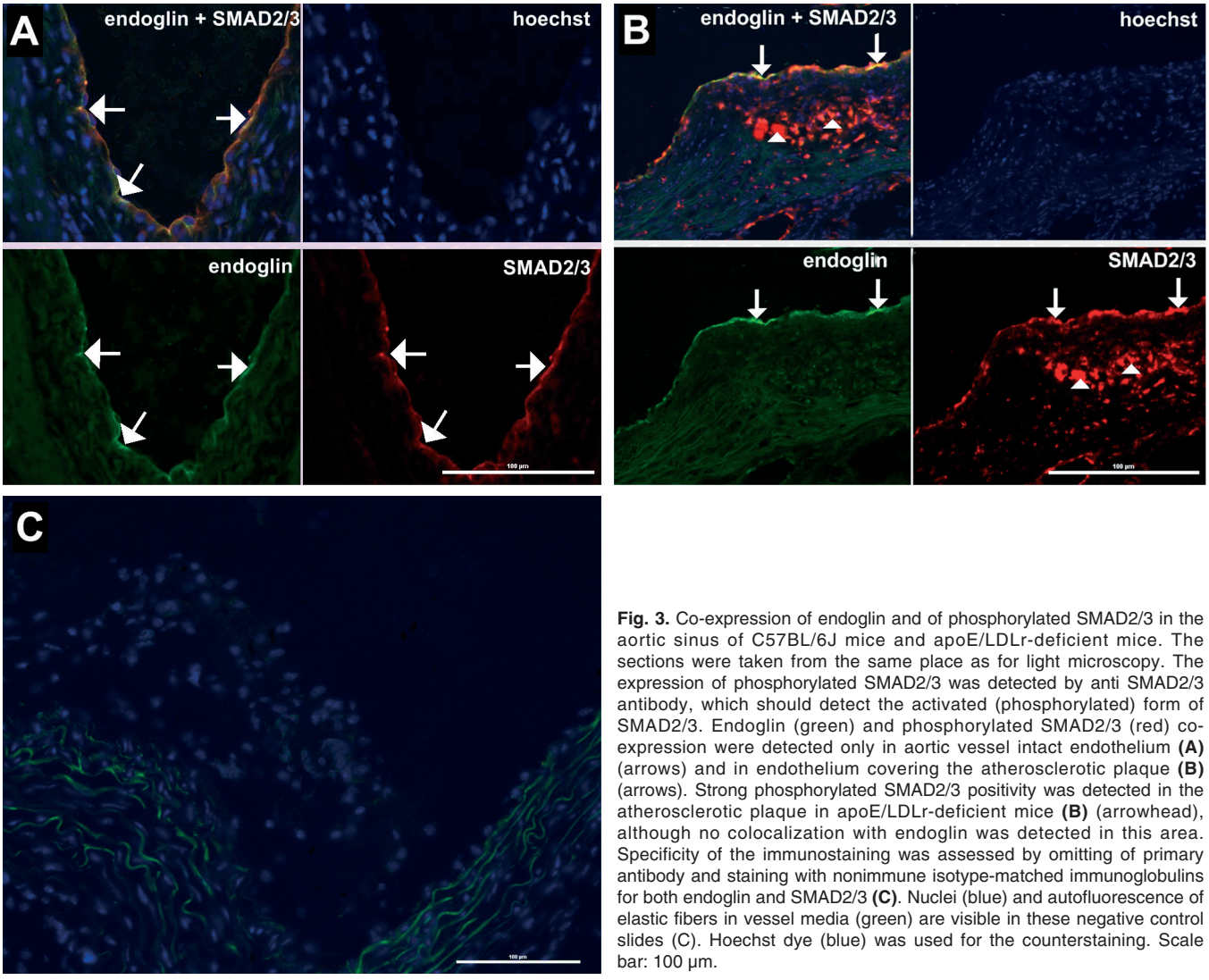

Fig. 3. Co-expression of endoglin and of phosphorylated SMAD2/3 in the aortic sinus of C57BL/6J mice and apoE/LDLr-deficient mice. The sections were taken from the same place as for light microscopy. The expression of phosphorylated SMAD2/3 was detected by anti SMAD2/3 antibody, which should detect the activated (phosphorylated) form of SMAD2/3. Endoglin (green) and phosphorylated SMAD2/3 (red) co-expression were detected only in aortic vessel intact endothelium **(A)** (arrows) and in endothelium covering the atherosclerotic plaque **(B)** (arrows). Strong phosphorylated SMAD2/3 positivity was detected in the atherosclerotic plaque in apoE/LDLr-deficient mice **(B)** (arrowhead), although no colocalization with endoglin was detected in this area. Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for both endoglin and SMAD2/3 **(C)**. Nuclei (blue) and autofluorescence of elastic fibers in vessel media (green) are visible in these negative control slides (C). Hoechst dye (blue) was used for the counterstaining. Scale bar: 100 µm.

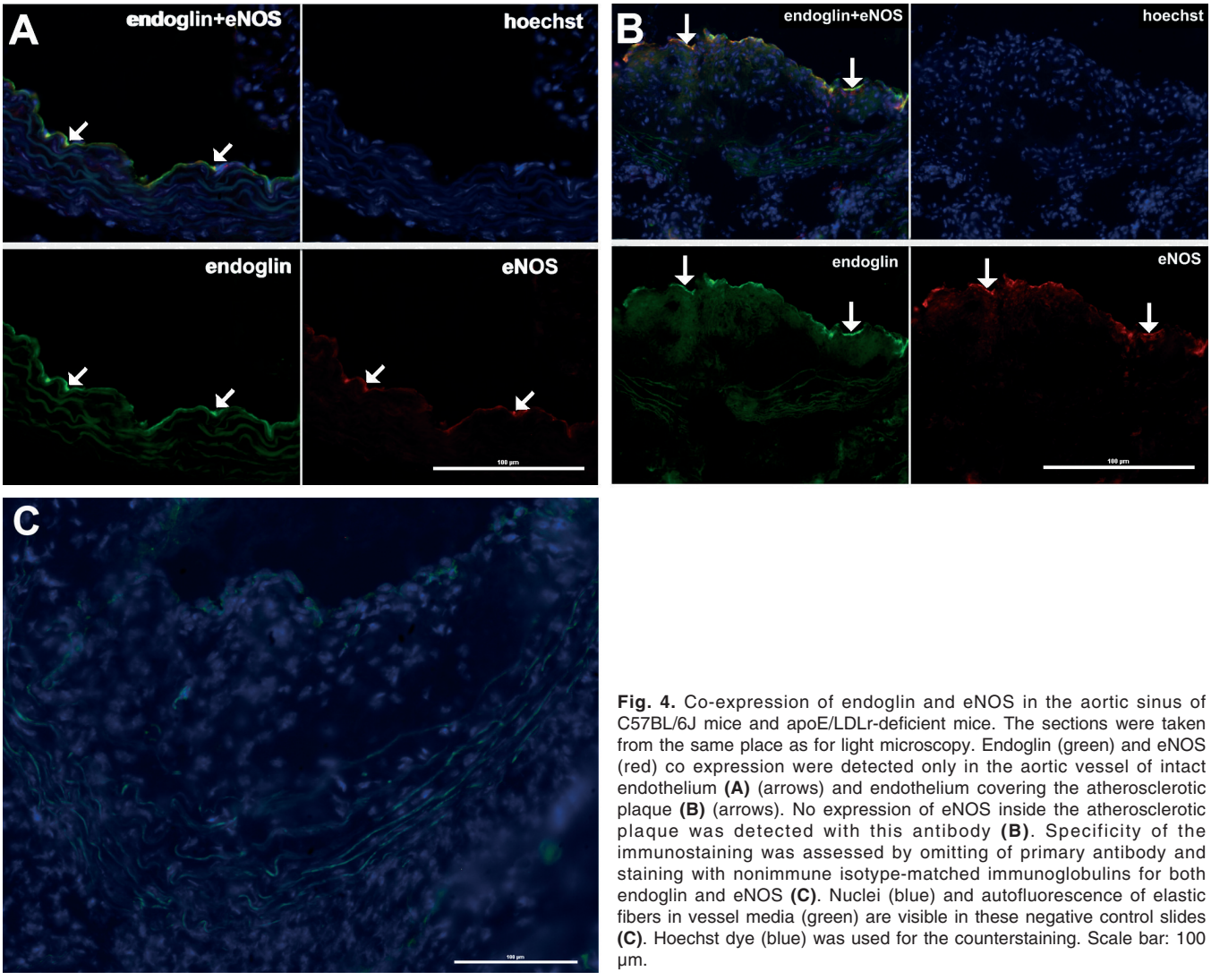

Fig. 4. Co-expression of endoglin and eNOS in the aortic sinus of C57BL/6J mice and apoE/LDLr-deficient mice. The sections were taken from the same place as for light microscopy. Endoglin (green) and eNOS (red) co expression were detected only in the aortic vessel of intact endothelium **(A)** (arrows) and endothelium covering the atherosclerotic plaque **(B)** (arrows). No expression of eNOS inside the atherosclerotic plaque was detected with this antibody **(B)**. Specificity of the immunostaining was assessed by omitting of primary antibody and staining with nonimmune isotype-matched immunoglobulins for both endoglin and eNOS **(C)**. Nuclei (blue) and autofluorescence of elastic fibers in vessel media (green) are visible in these negative control slides **(C)**. Hoechst dye (blue) was used for the counterstaining. Scale bar: 100 μm.

e.g. with or without cholesterol, to elucidate whether the co-expression can be affected by cholesterol levels, which were also obviously very different in animals used in this study.

Despite the fact that these *in vitro* mechanistic studies are not available so far, taking the recent data together with the results demonstrated in this paper, we might propose that endoglin expression in vessels, especially endothelium, might be protective due to its ability to affect eNOS expression via the SMAD2 dependent pathway.

In conclusion, we demonstrate here for the first time that endoglin is co-expressed with SMAD2, phosphorylated SMAD2/3 and eNOS by aortic endothelium *in vivo* in normocholesterolemic C57BL/6J mice and in hypercholesterolemic apoE/LDLr-deficient mice. Therefore, we suggest that endoglin, SMAD2, phosphorylated SMAD2/3 and eNOS may be important in vessel endothelium homeostasis underlying their role in atherogenesis.

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