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The ankyrin repeat containing SOCS box protein 5: a novel protein associated with arteriogenesis

Kerstin Boengler,[a,*] Frederic Pipp,[a] Borja Fernandez,[a] Alexandra Richter,[b] Wolfgang Schaper,[a] and Elisabeth Deindl[a]

[a] *Dept. of Experimental Cardiology, Max-Planck-Institute for Physiological and Clinical Research, Benekestr. 2, D-61231 Bad Nauheim, Germany*
[b] *European Molecular Biology Laboratory, Meyerhofstr. 1, D-69117 Heidelberg, Germany*

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Abstract

Arteriogenesis, the growth of pre-existing collateral arteries, can be induced in rabbit by occlusion of the femoral artery. In order to identify and characterize genes differentially expressed during the early phase of arteriogenesis, cDNA of collateral arteries 24 h after femoral ligation or sham operation was subjected to suppression subtractive hybridization. We identified the ankyrin repeat containing SOCS box protein 5 (asb5) and cloned the rabbit full-length cDNA. Asb5 was demonstrated to be a single-copy gene. We localized the asb5 protein in vivo in endothelial and smooth muscle cells of collateral arteries as well as in satellite cells. Asb5 was significantly upregulated in growing collateral arteries on mRNA and protein level. The infusion of doxorubicin in rabbit led to a significant decrease of the asb5 mRNA. In summary, our data show that asb5 is a novel protein implicated in the initiation of arteriogenesis.

Keywords: Asb5; Arteriogenesis; Gene expression; SOCS box

Current options to treat vascular occlusive diseases consist of revascularization techniques such as percutaneous transluminal angioplasty or bypass surgery. However, in recent years more and more effort was made to unravel the molecular mechanisms of collateral artery growth (arteriogenesis) in order to find drugs to combat arterial occlusive disease by stimulating the growth of natural pre-existing small bypasses. Arteriogenesis can be induced in rabbits by occlusion of the femoral artery [1]. The collaterals develop, e.g., in the m. quadriceps (m.q.), a tissue not associated with hypoxia or ischemia [2]. More likely, arteriogenesis is induced by fluid shear stress, which triggers an activation of the endothelium. Consequently, monocytes adhere and invade in collaterals and secrete growth factors and cytokines promoting the active growth of collateral arteries characterized by the proliferation of endothelial cells (ECs) and smooth muscle cells (SMCs) [3]. Previous studies showed that collateral artery growth is enhanced by infusion of the monocyte chemoattractant protein 1 (MCP-1) or the transforming growth factor β1 (TGFβ1) [4,5].

In this study we used a combination of suppression subtractive hybridization (SSH) and DNA-microarrays to investigate the differential gene expression in growing collateral arteries 24 h after femoral ligation and identified the ankyrin repeat containing SOCS box protein 5 (asb5) mRNA to be upregulated in growing collateral arteries. The asb proteins belong to the SOCS box proteins (suppressors of cytokine signaling), which in addition to the SOCS box contain ankyrin motifs. Other members of the family comprise a SH2 domain (SOCS proteins), WD-40 repeats (wsb proteins), a SPRY domain (ssb proteins) or a GTPase domain (RAR-like proteins) instead of the ankyrin repeats [6]. The SOCS proteins are best characterized among the members of the SOCS box family and it was demonstrated that they are implicated in the negative regulation of cytokine signaling [7].

* Corresponding author. Fax: +49-6032-705-419.
E-mail address: k.boengler@kerckhoff.mpg.de (K. Boengler).

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In our study we investigated the asb5 expression in rabbit collateral arteries and the surrounding tissue. To study the genomic structure of the asb5 gene, Southern blot analysis was performed. MCP-1, TGF-β1 or doxorubicin (dox), the latter used in cancer therapy, was infused in rabbits and the effects on the asb5 mRNA level were investigated by Northern blot and Real Time PCR.

Methods

Animal model. Femoral artery-ligated New Zealand White rabbits were studied as previously described [1]. These procedures were performed in accordance with local guidelines and recommendations.

Tissue samples from rabbit organs, from collateral arteries, and from the m.q. devoid of collateral arteries were collected 6, 12, and 24 h after femoral ligation or sham operation ($n = 5$ per point of time), snap frozen in liquid nitrogen, and stored at −80 °C until further investigations.

Right femoral arteries of rabbits ($n = 3$ per group) were ligated and were randomly assigned to receive either MCP-1 (3 μg/kg; Peprotech), TGF-β1 (0,48 μg/kg; Peprotech), or dox (2mg/kg; Sigma) at constant flow rates of 10 μL/h via osmotic minipump (2 ML-1, Alza) for 24 h. The left femoral arteries were sham operated.

RNA isolation and SSH. Total RNA was isolated as described previously [8]. The mRNA of 10 μg total RNA of collateral vessels 24 h after femoral ligation or sham operation was amplified with the SMART-technique (SMART PCR cDNA Synthesis kit, BD-Clontech). SSH (PCR Select cDNA Subtraction Kit, BD-Clontech) was performed according to the manufacturer's protocol using the SMART cDNA derived from collateral arteries 24 h after femoral occlusion as tester and the cDNA of the 24 h sham operated animals as driver.

Cloning of the rabbit asb5 cDNA. The full-length asb5 cDNA was cloned using the SMART RACE cDNA Amplification Kit (BD-Clontech) and the following primers: 3′-RACE: 5′-CGGTGTTAGAA GAGAGCCGACCGT-3′; 5′-RACE: 5′-CCAGGAACCTTGTCCTT GAGTTACTCCG-3′. Plasmid DNA was sequenced on an A.L.F. DNA sequencer using the AutoRead 200 sequencing kit (Amersham Biosciences).

DNA-microarray. Forty-four genes from the forward subtracted cDNA library were selected for DNA microarray analysis. The preparation of the microarrays and the fluorescent probes as well as the hybridization were performed as described previously [9]. In short, PCR products were spotted in five replicates on glass slides using a GMS 417 Arrayer (Affymetrix). The Cy3 or Cy5 labeled fluorescent probes were synthesized using the T7-RNA amplification method from 5 μg total RNA of collateral arteries 24 h after femoral ligation or sham operation. The slides were scanned on a GMS 418 Array Scanner (Affymetrix) and analyzed using the ChipSkipper microarray data evaluation software.

Northern blot hybridization. Northern blot analysis on total RNA was performed according to standard procedures [10]. The 550 bp asb5 cDNA used for hybridization was derived from the forward subtracted cDNA library. The 18S rRNA levels used for normalization were detected employing a specific oligonucleotide [11].

Quantitative Real Time PCR. One microgram DNase treated total RNA was reverse transcribed using random nonamers (Amersham Biosciences). Quantitative PCR was performed on an iCycler (Biorad) using Platinum Taq Polymerase (Invitrogen), SybrGreen I (Molecular probes), 100 nM of each primer (asb5 forward: 5′-AGACCATATC ACTCCATTGCT-3′, asb5 reverse: 5′-GATTGCGTTTACATTAGCT CCA-3′, 18S forward: 5′-GGACAGGATTGACAGATTGATAG-3′, 18S reverse: 5′-CTCGTTCGTTATCGGAATTAAC-3′) and 1 μl first strand RT reaction from a serial 10^0–10^{-4} dilution. Three independent RT reactions were used and for each dilution four replicates were amplified. The protocol was 3 min 95 °C initial denaturation, 30 s 95 °C, 1 min 60 °C, 45 cycles. Melt curve analysis was performed to control a specific amplification. The asb5 mRNA was normalized to the 18S rRNA level.

Antibodies and Western blot analysis. Two synthetic peptides corresponding to the amino acids 1–12 (MSVLEESRPFAQC) and 63–77 (QGQGSWADRSPLHEA) of the asb5 amino-acid sequence were used to immunize rats and the final serum was affinity purified (Eurogentec). Cytoplasmic protein extracts were isolated as described previously [10], separated on a 4–12% Bis–Tris gel (Invitrogen), and probed with the anti-asb5 antibody. Immunoreactivity was visualized using the ECL detection system (Amersham Biosciences).

Immunohistochemistry. Five or 10 μm cryosections of rabbit m.q. 24 h after femoral occlusion were fixed and the endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide. After blocking, the sections were incubated with an anti-asb5 antibody. Signals were detected by peroxidase-conjugated anti-rabbit IgG and 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin/eosin (H.E.). Photomicrographs were obtained with a Leica DC 200 video camera.

Southern blot hybridization. Genomic DNA from rabbit liver was isolated according to standard procedures [10]. Twenty microgram of genomic rabbit DNA digested with different restriction enzymes was hybridized with an asb5 specific cDNA probe.

Quantification and statistical analysis. In general, the values of the sham operated animals were set as 100%. The signals of Northern, Western, and Southern blots were detected and quantified with a Storm Imaging System (Molecular dynamics) using ImageQuant software. Statistical analysis was done by Student's t test. Values are presented as means ± SEM and were considered to be statistically significant (*) at $p < 0.05$.

Results

Cloning and characterization of the rabbit asb5 cDNA

Using suppression subtractive hybridization we compared the gene expression in rabbit collateral arteries 24 h after femoral ligation with collaterals from sham operated animals. The expression level of 44 genes from the forward subtracted library was analyzed by DNA-microarrays. We identified two significantly upregulated cDNA fragments, which both revealed significant homology to the mouse and human ankyrin repeat containing SOCS box protein 5 (asb5). Using 5′- and 3′-RACE-PCR we cloned the full-length cDNA, which represented the rabbit ortholog of asb5. The rabbit asb5 cDNA (Accession No. AY165034) consisted of 1637 bp and contained a single open reading frame of 329 amino acids. Conserved amino-acid elements included a putative transmembrane domain, six ankyrin repeat motifs, and a C-terminal SOCS-box (Fig. 1A). The comparison of the rabbit asb5 amino-acid sequence to those of the already known human and mouse asb5 amino acids demonstrated a 97% identity to the human and a 96% identity to the mouse asb5 protein (Fig. 1B).

Via Southern blot on digested rabbit genomic DNA, we detected one asb5 specific band for each of the enzymes or enzyme combinations. For the undigested

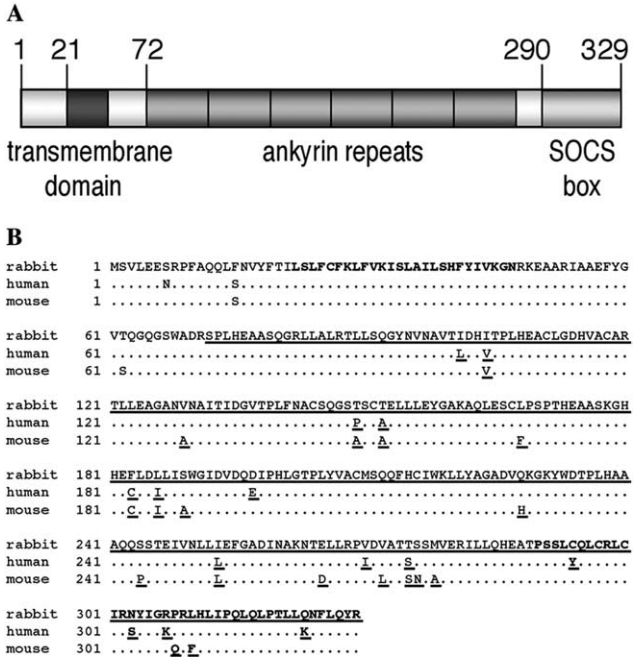

Fig. 1. The asb5 protein. (A) Schematic representation of the putative functional domains of the asb5 protein. (B) Alignment of the mouse, human, and rabbit asb5 amino-acid sequences. Amino acids of the transmembrane domain are printed in bold, the ankyrin repeats are underlined, and the SOCS box is printed in bold and underlined.

DNA, we obtained a signal at ~15 kb, confirming the integrity of the genomic DNA (Fig. 2).

Immunohistochemical data on rabbit m.q. harboring pre-existing arterioles showed that asb5 is localized in the cytoplasma of ECs and SMCs of arteries as well as in the ECs of capillaries (Figs. 3A,B,D,E). Furthermore, satellite cells of myocytes stained positive for asb5, whereas the myocytes themselves were negative (Fig. 3C).

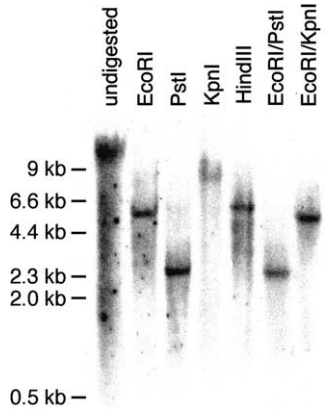

Fig. 2. Southern blot analysis of the asb5 gene. Rabbit genomic DNA, undigested or digested with distinct restriction enzymes was hybridized with an asb5 specific probe. The positions of the DNA marker are indicated.

Asb5 is upregulated in growing collateral arteries

Via Northern blot analysis we found an expression of the asb5 mRNA in rabbit skeletal muscle and heart, but not in brain, lung, kidney, thyroid gland, uterus, or liver (Fig. 4A). In the m.q. (devoid of collaterals) isolated 6, 12, and 24 h after the surgical procedure we observed a strong signal for the asb5 mRNA, but no differences between experimental and sham operated animals (Fig. 4B). Results of Real Time PCR (Fig. 4D), which was performed for quantification, showed a significant upregulation of the asb5 mRNA in collateral arteries 6 h (240%), 12 h (210%), and 24 h (230%) after femoral ligation and confirmed Northern blot results (Fig. 4C).

Western blot analysis on cytosolic extracts of collaterals isolated 24 h after the surgical procedure revealed a single immunoreactive band (~38 kDa) that showed 3-fold values in its density in experimental compared to sham operated animals (Fig. 5).

Infusion of dox in rabbit collateral arteries for 24 h resulted in a significant downregulation of the asb5 mRNA as shown by Northern blot (factor 0.7) and Real Time PCR (factor 0.5) (Fig. 6). The infusion of MCP-1 or TGF-β1, however, showed no effect on the asb5 mRNA level (data not shown).

Discussion

Analyzing the differential gene expression in growing collateral arteries, we identified asb5, a protein not described in any functional context until now. We cloned the rabbit ortholog of asb5, which is a single-copy gene. The asb5 protein was localized in ECs and SMCs of arteries and in the endothelium of capillaries as well as in the satellite cells of myocytes. In a rabbit model of arteriogenesis, we found asb5 to be upregulated on mRNA and on protein level at early stages of collateral artery growth. Furthermore, our data evidenced that the asb5 mRNA was downregulated by dox in vivo.

The objective of this study was to identify and characterize genes differentially expressed during the initiation of arteriogenesis. Using in combination SSH and low-density DNA-microarrays we compared the gene expression in collateral arteries 24 h after femoral ligation with sham operated animals and identified the asb5 mRNA to be upregulated in this assay.

Asb5 is a member of the asb family, which is characterized by a non-conserved N-terminus, a various number of ankyrin repeats—between three (asb14) and 15 (asb2)—as well as a C-terminal SOCS box [12,13]. In this study we cloned the rabbit asb5 cDNA, which comprised of 1637 bp and contained an open reading frame of 329 amino acids. At the bp 38–40 an in-frame stop-codon was identified, suggesting that we obtained the complete asb5 encoding cDNA. Analysis of the asb5

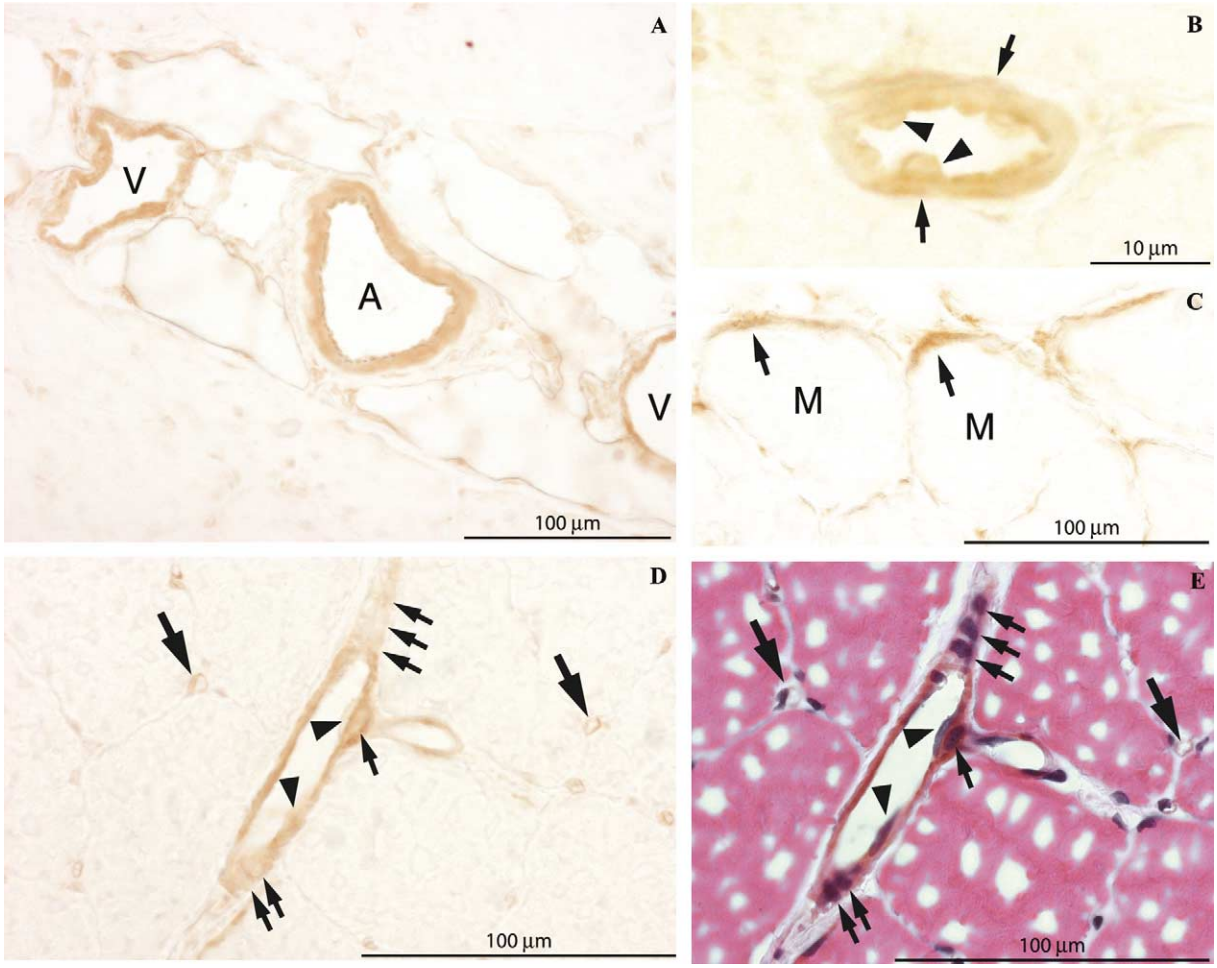

Fig. 3. Immunoperoxidase localization of asb5 (A)–(D) and H.E. counterstaining (E) of rabbit m.q. (A) Asb5 immunoreactivity was found in the wall of collateral arteries (A) and veins (V). (B) Both ECs (arrowheads) and SMCs (arrows) were positive for asb5. (C) Some satellite cells (arrows) were positive for asb5. Myocytes (M) were negative. (D, E) asb5 stained the cytoplasm of ECs and SMCs of arteries (arrowheads and small arrows, respectively). Capillaries (big arrows) were also positive. Scale bar: 100 µM.

amino-acid sequence revealed a putative transmembrane domain (amino acids 21–43), six ankyrin repeats (amino acids 72–269), and the SOCS box at the C-terminus of the protein (amino acids 290–329). Comparison of the rabbit, human, and mouse asb5 amino-acid sequences revealed a more than 96% identity, indicating that asb5 is highly conserved during evolution. The ankyrin motifs were identified in more than 400 proteins, exerting different functions such as regulation of transcription, organization of the cytoskeleton, or control of developmental processes. On cellular level, they mediate protein–protein interactions [14]. The C-terminus of asb5 contains the SOCS box, a 39 amino-acid motif first identified in the proteins SOCS1-3, which also contain a SH2 domain [15]. The SOCS proteins function in a negative feedback mechanism by inhibiting cytokine induced signal transduction. The complexes of SOCS proteins and their bound signaling molecules are targeted for the degradation pathway via interaction with the elongins B and C [16].

The family of the asb proteins comprises of 18 members [13]. In order to investigate the genomic structure of the rabbit asb5 gene, Southern blot experiments were performed. Data showed only one specific signal after hybridization of the asb5 probe to rabbit genomic DNA, digested with distinct restriction enzymes, indicating that asb5 is a single-copy gene. We therefore conclude that the asb family represents a protein family rather than a gene family.

We showed an expression of the rabbit asb5 mRNA by Northern blot hybridization in heart and skeletal muscle, but not in brain, lung, kidney, thyroid gland, uterus, or liver. In a previous study [12], the murine asb5 mRNA was also detected in testis and bone shaft. The expression pattern of the asb5 mRNA is similar to those of asb2 and asb10, both of which are predominantly expressed in heart and muscle [12].

By Northern blot analysis and Real Time PCR we demonstrated an upregulation of the asb5 mRNA in collateral arteries 6, 12, and 24 h after femoral ligation,

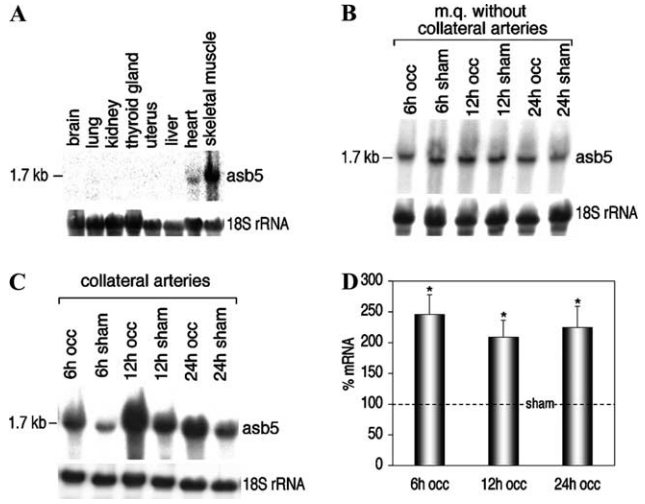

Fig. 4. Characterization of the asb5 mRNA level. Total RNA of rabbit organs (A), of the m.q. devoid of collaterals (B), and of collaterals (C) 6, 12, and 24 h after femoral occlusion (occ) or sham operation was hybridized with an asb5 specific probe (top). The blots were rehybridized with an 18S rRNA specific probe (A–C; bottom). (D) Results of Real Time PCR on asb5 mRNA levels in collateral arteries.

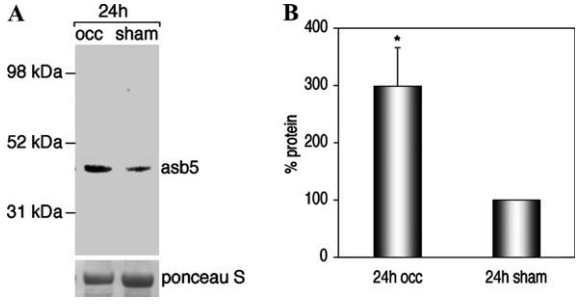

Fig. 5. Asb5 protein level in collaterals. (A) Western blot analysis of asb5 on cytosolic extracts of collateral arteries 24 h after femoral occlusion (occ) or sham operation. To control equal loading, a representative ponceau S protein staining is shown. (B) Quantification of the asb5 protein expression.

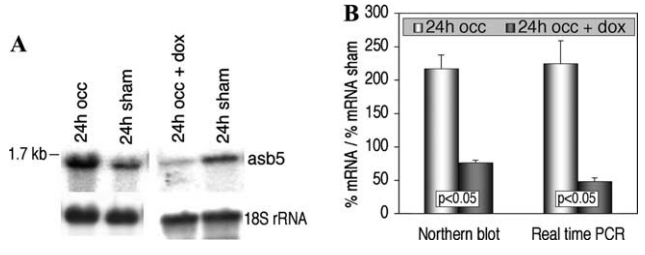

Fig. 6. Modulation of the asb5 mRNA expression in vivo. (A) Northern blot analysis of asb5 mRNA levels in collaterals 24 h after occlusion and after occlusion plus infusion of dox in rabbit (top). The blot was rehybridized with an 18S rRNA specific probe (bottom). (B) Quantification of Northern blot and Real Time PCR results of asb5 mRNA levels in collaterals.

although a minor increase was seen 12 h after sham operation. This indicates an influence of the surgery itself on the asb5 mRNA level. In tissue samples of the rabbit m.q. devoid of collateral arteries, we detected no differential expression of the asb5 mRNA, suggesting that the asb5 mRNA is specifically upregulated in collateral arteries.

In order to analyze the specificity of the antibody generated against asb5, Western blot analysis was performed. In cytosolic extracts of rabbit collateral arteries only one specific signal of ~38 kDa was detected, corresponding to the deduced molecular weight of ~36 kDa. Results showed a 3-fold increase of the asb5 protein level in collaterals 24 h after femoral ligation compared to sham operation.

So far, the expression of asb mRNAs was described only on the RNA level for different organs. By immunohistochemistry we showed a localization of the asb5 protein in the ECs of capillaries, as well as in ECs and SMCs of arteries. Furthermore, our data evidenced that asb5 is localized in some satellite cells of myocytes, whereas myocytes themselves do not express asb5.

The exact functions of individual asb proteins are not well characterized and up to now no association of asb proteins with vessel growth has been described. Asb1 knockout mice appeared normal, only the testis displayed a reduced spermatogenesis [12]. Previous studies showed an induction of the asb2 mRNA level by retinoic acid, leading to growth inhibition and chromatin condensation [17,18]. The exact function of asb5 in arteriogenesis remains unclear. We speculate, however, that asb5 interacts via the SOCS box with the elongin BC complex, thereby coupling itself and its substrate to the proteasomal degradation pathway, as it was shown for asb2 [16]. It is unlikely that substrates of asb5 are members of the JAK/STAT pathway since asb5 does not contain the SH2 domain necessary for protein–protein interaction [19]. Asb5 rather contacts other signaling molecules via its ankyrin repeats.

In order to influence the asb5 mRNA level in vivo, MCP-1, TGF-β1, or dox was infused in rabbits and the asb5 mRNA level was investigated. During arteriogenesis, MCP-1 enhances the adherence of monocytes and TGF-β1 functions in activating monocytes, thereby increasing their adhesion and invasion and finally the secretion of growth factors [4,5]. Our results demonstrate no alteration of the asb5 mRNA level after infusion of MCP-1 or TGF-β1, suggesting that in collateral artery growth asb5 is not a downstream target of these factors, but is involved in other signaling pathways. Results of both Northern blot and Real Time PCR showed that the infusion of dox, widely used in cancer therapy, decreased the asb5 mRNA level in collateral arteries compared to femoral occlusion alone. It was shown that dox mediates its effects by generating H_2O_2, thereby inducing apoptosis [20]. In addition, dox was demonstrated to inhibit angiogenesis [21].

From our study we conclude that asb5 represents a novel protein associated with the initiation of collateral artery growth presumably by interfering with the signal transduction cascade.

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