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Basement Membrane Remodeling in Skeletal Muscles of Patients with Limb Ischemia Involves Regulation of Matrix Metalloproteinases and Tissue Inhibitor of Matrix Metalloproteinases

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Key Words
Basement remodeling · Limb ischemia · Matrix metalloproteinase · Peripheral arterial disease · Skeletal muscle · Tissue inhibitor of matrix metalloproteinase

Abstract
Background/Aim: Because the pericapillary basement membrane in skeletal muscles of patients with chronic critical limb ischemia (CLI) is thickened, we determined the expression patterns of genes involved in collagen metabolism, using samples from 9 CLI patients, 4 patients with acute limb ischemia and 4 healthy controls. ***Methods:*** Gene array analysis, quantitative RT-PCR and semiquantitative grading of immunohistochemical reactivity were performed to determine mRNA/cDNA and protein concentrations. ***Results:*** In CLI patients compared to controls, cDNA levels of matrix metalloproteinase (MMP)-9 and MMP-19 were higher, collagen type IV chains A1 and A2, tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 were similar and MMP-2 were lower. On the protein level, MMP-2, MMP-9, MMP-19 and TIMP-1 were more abundantly expressed. In skeletal muscles from patients with acute limb ischemia, cDNA and protein levels of MMP-9, MMP-19, collagen type IV chains, TIMP-1 and TIMP-2 were high. MMP-2 was elevated at the protein but decreased on the cDNA level. ***Conclusion:*** Expression of basement membrane components in skeletal muscles of CLI and acute limb ischemia patients is altered, possibly contributing to the pathogenesis of peripheral arterial disease.

Introduction

Human peripheral arterial occlusive disease (PAD) is a consequence of chronic ischemia, which is caused by a continuously developing atherosclerotic stenosis or obstruction of large arteries in the pelvis or leg. Peripheral blood flow is thereby reduced, which gives rise to a chronic deficiency in the tissue supply of nutrients and oxygen. Hypoxia ensues and results in chronic critical limb ischemia (CLI) within the affected skeletal muscles as observed in patients with intermittent claudication [1]. If left untreated for many years, this condition leads to the development of gangrene and ulcers [2]. In extreme cases, the damaged extremity must be amputated.

A severe ischemic event caused by the sudden occlusion of a supplying artery can also compromise the perfu-

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sion of the limb and thus jeopardize its viability. Such acute cases of ischemia can occur both in asymptomatic individuals (acute ischemia) or be superimposed within the predamaged arteries of PAD patients (acute-on-chronic ischemia). In the absence of preexisting collaterals, and as a consequence of this circumstance, cases of subacute ischemia tend not only to be unrelenting but also to develop into a very severe condition.

Mainly in rodents, several animal models have been established to investigate chronic hind limb ischemia. In these experimental setups, the blood supply to the hind limb is surgically suppressed by ligation of the femoral, the deep femoral and/or the external iliac arteries. As far as we are aware, such surgically induced ischemia is continued for maximally 63 days [3]. Hence, these experimental animal models mimic acute limb ischemia rather than the slow, progressively deteriorating state manifested in patients with CLI. Furthermore, a recent study has shown that animal models of skeletal muscle ischemia involve more collateral arteries, a higher blood flow and less necrosis and inflammation than is characteristic of acute hind limb ischemia [4]. This observation indicates that the animal models imitate clinically subacute rather than acute hypoxic conditions.

Recently, we reported the capillaries in skeletal muscles of CLI patients to be surrounded by an enlarged basement membrane containing abundant smooth muscle cell actin-immunoreactive cells [5]. This latter observation indicates that the capillaries transformed into functional arterioles (arteriolization). This vascular remodeling process was observed in patients with intermittent claudication, which suggests that it is associated with the early development of PAD. So far, the molecular mechanisms underlying basement membrane thickening have not been elucidated.

In general, the appearance of a basement membrane reflects the equilibration established between the biosynthesis and degradation of its components [6]. The major component of this entity is collagen type IV, which has a relatively long half-life [review in 7]. The interplay between matrix metalloproteinases (MMPs) and their natural antagonists, namely the tissue inhibitor of metalloproteinases (TIMPs), mainly governs the turnover of the basement membrane [review in 8]. Both MMPs and TIMPs contribute to the pathogenesis of major cardiovascular diseases such as atherosclerosis and restenosis [9]. Although the expression profiles of MMPs and TIMPs in healthy skeletal muscle have been thoroughly characterized [review in 7, 10, 11], the impact of these molecules on diseased human tissue has not been investigated.

To ascertain whether the thickened basement membrane observed in CLI patients goes along with changes in the molecular organization, we quantified the expression levels not only of its major and minor components, but also of molecules involved in its turnover at both the mRNA level by means of a gene array analysis and real-time RT-PCR as well as at the protein level by semiquantitative immunohistochemistry. Furthermore, to investigate whether the arteriolization of capillaries also occurs under acute ischemic conditions, the same approach was applied to the skeletal muscles of patients suffering from acute limb ischemia.

Materials and Methods

Skeletal Muscle Samples

Human skeletal muscles were obtained from patients undergoing lower limb surgery at the University Hospital Bern (Inselspital) between 2003 and 2004. Samples were derived from lower limbs that had been amputated for acute ischemia in 4 cases and CLI in 9 cases. Material derived from patients undergoing free-flap muscle transposition in 4 asymptomatic patients served as controls. All symptomatic CLI patients had a history of PAD and a stable manifestation of this condition for at least 2 months prior to tissue sampling. PAD was defined according to the 'Second European consensus document on chronic critical leg ischemia'. Our cohort of patients embraced Fontaine stages III and IV [12]. Demographic data relating to the patients and the controls are presented in table 1. The Ethics Committee of the University Hospital Bern approved the study protocol. All patients gave written informed consent.

Unless otherwise stated, skeletal muscle samples collected were derived from the proximal and distal parts of the tibialis anterior and the gastrocnemius muscles as indicated in table 1.

RNA Isolation

Total RNA was extracted from the skeletal muscle samples, which had been stored in RNAlater (Qiagen, Hilden, Germany) using the RNeasy fibrous tissue mini kit (Qiagen) according to the manufacturer's instructions. Prior to precipitation with ethanol, traces of DNA were removed by treatment with DNase I (Qiagen). After elution with RNase-free water, the final concentrations of RNA were determined in a spectrophotometer ND-1000 (Nano Drop, Wilmington, Del., USA).

Gene Array Analysis

The gene array analysis was performed on skeletal muscle samples derived from 2 healthy individuals, 4 patients with acute ischemia and 6 CLI patients as indicated in table 1. Altogether, 12 samples were applied to human U133A GeneChips (Affymetrix, Santa Clara, Calif., USA).

Biotin labeling of RNA was performed as described in the Expression Analysis Technical Manual (Affymetrix) with minor modifications as indicated below. To synthesize single-stranded cDNA, 5 μg of total RNA was mixed with oligo-dT and incubated with SuperScript II reverse transcriptase (Invitrogen, Gaithers-

Table 1. Summary of demographic data relating to asymptomatic control individuals and patients suffering from PAD with acute limb ischemia or CLI

Patient	Diagnosis	Age years	Sex	Diabetes type II	Hyper-tension	Hypercho-lesterolemia	Smoker	Samples	Gene array	qRT-PCR	IHC
GM	healthy	79	f	no	no	no	yes	TA-prox.	×	×	×
MG	healthy	53	m	no	no	no	yes	GC-mid		×	×
TE	healthy	58	m	no	yes	no	yes	GC-prox.		×	×
UN	healthy	66	f	no	yes	yes	no	TA-prox.	×	×	
BW	acute (11 days)	44	m	yes	yes	yes	yes	GC-prox.		×	×
								GC-dist.		×	×
								TA-prox.		×	×
								TA-dist.		×	×
RE	acute (10 days)	72	m	no	yes	yes	yes	GC-prox.	×	×	×
								GC-dist.		×	×
SE	acute-on-chronic (10 days)	71	f	yes	no	yes	no	GC-prox.		×	×
								GC-dist.		×	×
								TA-prox.	×	×	×
								TA-dist.		×	×
SW	acute ischemic syndrome	62	m	no	yes	no	yes	TA-prox.	×	×	
								TA-prox.	×	×	
AH	CLI (IV)	87	m	no	yes	no	yes	GC-prox.	×	×	×
BA	CLI (III)	79	m	no	yes	yes	yes	GC-prox.		×	×
								GC-dist.		×	×
BE	CLI (IV)	72	f	no	yes	yes	yes	GC-prox.		×	×
								GC-dist.		×	×
								TA-prox.			×
								TA-dist.		×	×
FB	CLI (IV)	61	m	yes	yes	yes	yes	GC-prox.		×	×
								GC-dist.		×	×
GJ	CLI (IV)	88	m	no	no	yes	yes	GC-prox.	×	×	×
								GC-mid.		×	×
GR	CLI (IV)	85	f	no	yes	no	no	TA-dist.	×	×	×
RR	CLI (IV) Buerger's disease	31	m	no	no	no	yes	GC-dist.		×	×
								GC-mid.		×	×
								TA-prox.	×	×	×
								TA-dist.	×	×	×
S.H.	CLI (IV)	80	m	yes	yes	no	no	GC-prox.		×	×
								GC-dist.		×	×
Z.E.	CLI (IV)	62	m	no	no	yes	yes	TA-prox.	×	×	

Information about the samples collected and the analytical methods applied is also provided. IHC = Immunohistochemistry; f = female; m = male; TA = tibialis anterior muscle; GC = gastrocnemius muscle; prox. = proximal; dist. = distal.

burg, Mass., USA) at 42°C for 1 h. After synthesizing double-stranded cDNA following the SuperScript choice kit protocol (Invitrogen) and using the *Escherichia coli* DNA ligase (2 U/μl), the *E. coli* DNA polymerase (5 U/μl) and RNase H (1 U/μl) supplied by Roche Diagnostics (Penzberg, Germany), the material was extracted with phenol-chloroform-isoamyl alcohol and coprecipitated with 10 μg glycogen and 0.5 vol of 7.5 M ammonium acetate and 2.5 vol of ethanol. All cDNA was used for in vitro transcription with the BioArray HighYield RNA Transcript (T7) labeling kit (Enzo, New York, N.Y., USA) to synthesize RNA in the pres-

ence of biotin-conjugated ribonucleotide analogs. Approximately 53 μg of labeled cRNA from each reaction was purified with RNeasy mini spin columns, and roughly 300 ng was analyzed on RNA Nano 6000 Chips. The cRNA targets were incubated at 94°C for 35 min and the resulting fragments of 50–150 nucleotides were again monitored using a bioanalyzer. All synthesis reactions were carried out in a PCR machine (T1 thermocycler; Biometra, Göttingen, Germany) to ensure the highest possible degree of temperature control. The hybridization cocktail (220 μl) containing fragmented biotin-labeled target cRNA at a final concentration of 0.05 μg/μl was transferred into human U133A GeneChips (Affymetrix) and incubated at 45°C on a rotator in a hybridization oven 640 (Affymetrix) for 16 h at 60 rpm. The arrays were washed and stained by using a streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, Oreg., USA). To increase the signal strength, the antibody amplification protocol was used (EukGE-WS2v4; Affymetrix Expression Analysis Manual).

The GeneChips were scanned and raw expression values were computed from the images using the Affymetrix Microarray Analysis Suite, version 5.0. RMA-Express was applied for normalization of raw expression data (CEL files). RMA-normalized expression values of the arrays were then z-score normalized. A z-score is computed by subtracting the probe's profile mean value from its expression value and subsequently dividing it by its standard deviation. For a subset of 14 genes, average expression values were computed for each patient group. From these, a heat map was computed using Mayday [13]. Note that because of the z-score normalization of the genes' profiles, the heat map visualizes relative and not absolute expression changes between patient groups. Since absolute height of expression is not visualized, expression levels are not comparable between genes.

Quantitative RT-PCR

For validation of data from gene array analysis, the expression levels of MMP-2, MMP-9 and GAPDH as examples were quantified in 35 samples (table 1) by real-time RT-PCR using TaqMan low-density arrays (Applied Biosystems, Foster City, Calif., USA), which were loaded with gene-specific probes and primers spanning at least one exon-intron boundary.

RT was performed at 37°C for 2 h using a 4-μg sample of total RNA, a 1-μg aliquot of random primers and 250 U of Multiscript reverse transcriptase (Applied Biosystems). In accordance with the instructions of the manufacturer, a 1.5-μl sample of cDNA, dissolved in 48.5 μl of RNase-free water, was mixed with 50 μl of TaqMan universal PCR master mix (Applied Biosystems) prior to loading on 7900HT microfluidic cards. Thermal cycling was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems) in conjunction with the following program: 2 min at 50°C (activation of uracil-DNA glycosylase), 10 min at 95°C (denaturation), 40 cycles at 95°C (15 s) and annealing/extension at 60°C (60 s).

Real-time quantitative RT-PCR (qRT-PCR) data were monitored using SDS 2.1 software (Applied Biosystems). The results were quantified using a manually assigned baseline to record the cycle thresholds (Ct). According to the slopes of the logarithmic linear face of the amplification curves, PCR efficiency was about 95% in all cases.

The expression levels of mRNA were calculated using the formula:

$$n = 2^{(\text{Ct of gene of interest} - \text{Ct of GAPDH})} \times 1{,}000.$$

Light and Electron Microscopy

For light and electron microscopy, samples of skeletal muscles were fixed in Karnowsky's solution [2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4] at 4°C for several days. One-micrometer-thick sections for light microscopy and 80- to 90-nm-thick ultrathin sections for electron microscopy were prepared and stained as previously described [5].

Immunohistochemistry

Samples of skeletal muscle were fixed in 4% (v/v) paraformaldehyde in PBS for 4 h at 4°C, washed in 10% (w/v) sucrose for 2 h and then stored in 70% (v/v) ethanol at –20°C until use. Immunohistochemistry on paraffin-embedded sections was performed as previously described [5]. Monoclonal antibodies against MMP-2, MMP-3 and MMP-9 were obtained from Neomarkers (Fremont, Calif., USA). Monoclonal antibody against MMP-19 was kindly provided by Dr. R. Sedlacek, Kiel, Germany [14]. Anti-TIMP-1 and anti-TIMP-2 monoclonal antibodies were purchased from Oncogene (Cambridge, Mass., USA). The monoclonal antibody against macrophage/monocyte-specific CD163 (corresponding to rat ED2-scavenger receptor expressed in intermediate and late inflammatory stages in skeletal muscles [15]) was acquired from Acris (Hiddenhausen, Germany). The collagen type IV antibody was obtained from Sigma (Buchs, Switzerland). Negative controls were prepared using nonspecific antisera. Immunoreactivity was detected by means of avidin-biotin horseradish-peroxidase complex (Dako, Glostrup, Denmark), using 3-amino-9-ethylcarbazole (Sigma) as a substrate.

Semiquantititive scoring for MMPs and TIMP-1 expression was undertaken by 2 researchers, who independently graded the intensity of immunohistochemical reactivity according to a 5-step scale, with 0 = no detectable vascular immunoreactivity, 1 = fewer than 20%, 2 = fewer than 50%, 3 = fewer than 80% and 4 = more than 80% of the potentially immunoreactive sites being positive.

Statistics

ANOVA was performed to compare the differences in age between the patient cohorts. The gene array data were normalized using GADA as housekeeping gene. A two-class Student t-test with Welch modification and the Rank product method [16] were applied to detect differentially expressed genes.

GAPDH-normalized data relating to the cDNA levels of MMP-2 and MMP-9 obtained by qRT-PCR, as well as the semiquantitative immunohistochemical findings were expressed as means ± SD.

qRT-PCR-normalized expression values were compared with normalized gene array expression values. ANOVA and t-test analysis were performed to compare the cDNA expression levels determined by qRT-PCR in both groups of PAD patients compared to asymptomatic control patients. $p \leq 0.05$ was considered statistically significant. The Pearson correlation coefficient for the cDNA concentrations of MMP-2 and MMP-9 in those 12 samples that had been subjected to both gene array analysis and qRT-PCR was then computed.

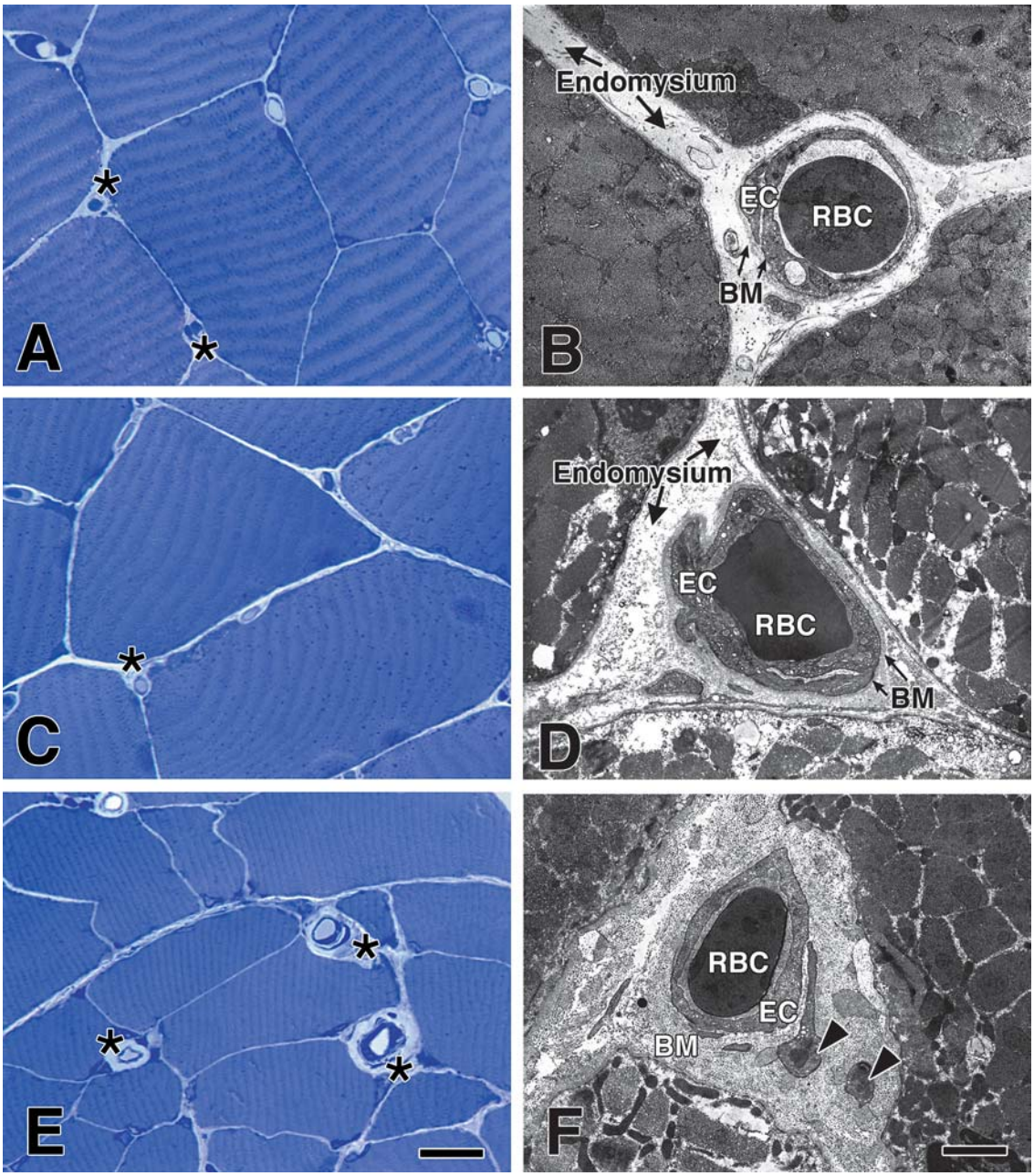

Fig. 1. Ultrastructure of the basement membrane around capillaries in skeletal muscle of patients with CLI and acute limb ischemia. **A**, **C**, **E** Representative light micrographs of toluidine blue-stained 1-μm-thick sections. In sections from asymptotic control patients (**A**) and patients with acute limb ischemia (**C**), the basement membrane around skeletal muscle capillaries appeared unstained and discrete, whereas in those with CLI (**E**), it is of considerable thickness. **B**, **D**, **F** Transmission electron microscopy revealed the basement membrane around skeletal muscle capillaries in control patients (**B**) to contain small amounts of nonfibrillar collagenous material. The microvascular basement membrane was slightly enlarged in samples from acute limb ischemia patients (**D**). In CLI patients (**F**) the pericapillary basement membrane was significantly extended and contained various types of cells (arrowheads). Note the different electromicroscopic appearance of densely packed collagen type IV specific for basement membranes and the loosely organized fibrillar collagen type I of the endomysium. * = Pericapillary basement membrane; BM = basement membrane; EC = endothelial cell; RBC = red blood cell. **E** Scale bar = 20 μm. **F** Scale bar = 5 μm.

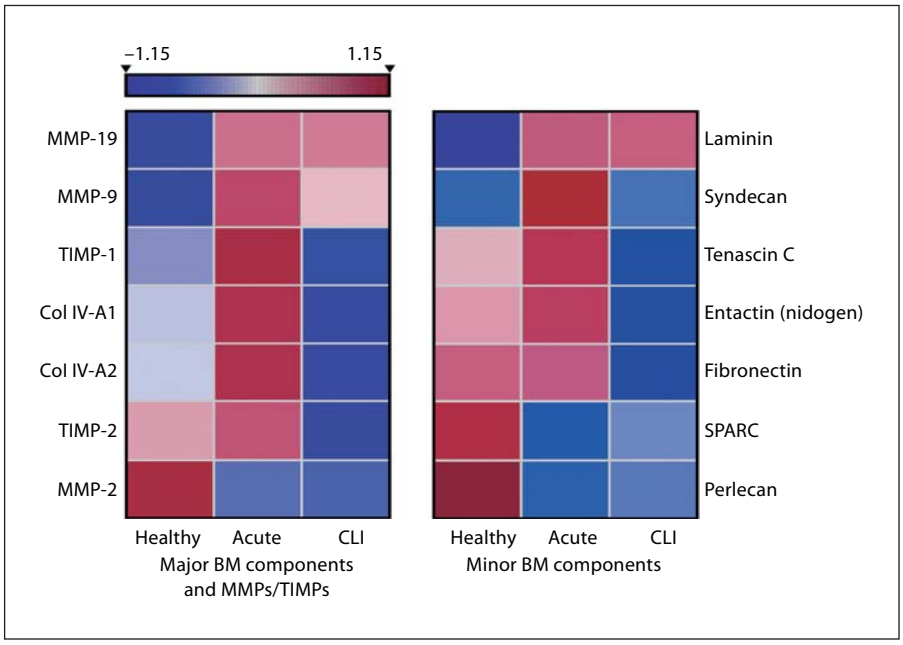

Fig. 2. Heat maps based on the gene array analysis summarizing the quantitative expression patterns of genes involved in basement membrane remodeling. The cDNA extracted from the skeletal muscles of healthy controls (2 samples) and patients with acute limb ischemia (4 samples) and CLI (6 samples) was subjected to a gene array analysis using human U133A GeneChips (Affymetrix). The mean gene expression value for each patient group was computed and used to calculate the heat map, which thus reflects the cDNA concentration of genes involved in basement membrane remodeling. Expression profiles are color coded (red = high expression level; blue = low expression level). Note that relative and not absolute expression changes between patient groups are shown due to the z-score normalization of the gene profiles. Since absolute height of expression is not visualized, expression levels are not comparable between genes. BM = Basement membrane; Col = collagen.

Results

The skeletal muscle samples of the control group used in this study were derived from 4 patients with an age of 64 ± 11 years while the acute limb ischemia group consisted of 4 patients with an age of 63 ± 13 years and the CLI group of 9 patients with an age of 72 ± 18 years (table 1). These differences in age between the patient cohorts were statistically not significant (data not shown).

The structure of the basement membrane surrounding skeletal muscle capillaries was first examined by light microscopy using toluidine blue-stained 1-μm-thick sections. In asymptotic control patients (fig. 1A) and acute limb ischemia patients (fig. 1C), a vascular basement membrane was not detectable, while a sizeable unstained cuff consistently mantled the capillaries in CLI patients (fig. 1E). Electron microscopy analysis revealed the abluminal cuff around the skeletal muscle capillaries in acute limb ischemia patients to consist of small quantities of dense nonfibrillar collagen typical for basement membrane-specific collagen type IV (fig. 1D), which was not detected in samples from controls (fig. 1B). The collagenous basement membrane was significantly thicker in skeletal muscles of CLI patients, within which often various types of cells were embedded (fig. 1F).

Performing a gene array analysis, the mRNA/cDNA levels of several basement membrane components were compared in skeletal muscles derived from control individuals (2 samples) and patients with acute limb ischemia (4 samples) and CLI (6 samples). These findings were summarized in the form of a heat map for selected genes (fig. 2). Compared to the content in skeletal muscle samples of controls, the mRNA/cDNA levels for major basement membrane-specific collagen type IV chains A1 and A2 as well as TIMP-1 and TIMP-2 were significantly higher in samples of acute limb ischemia patients, and slightly lower than those in samples of CLI patients. The mRNA/cDNA concentrations for MMP-9 and MMP-19 were higher in samples derived from patients belonging

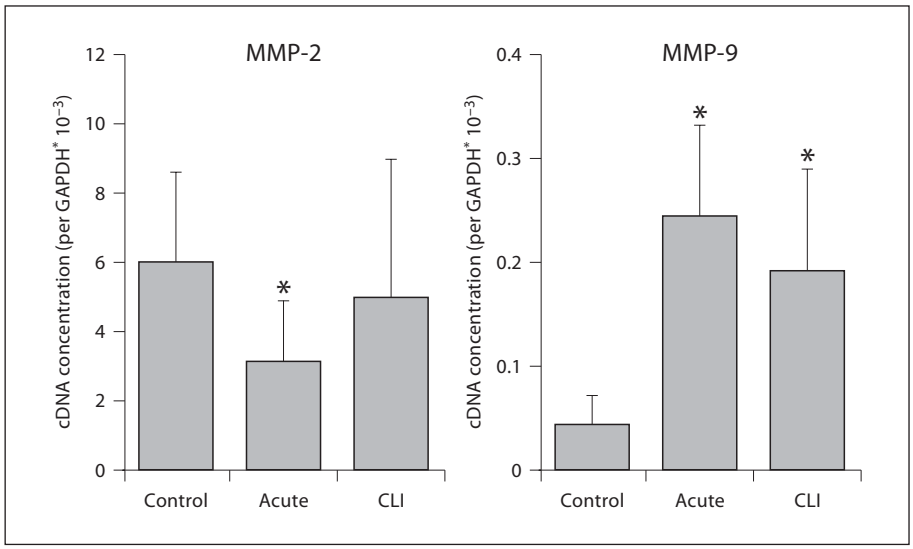

Fig. 3. Quantification of MMP-2 and MMP-9 levels by real-time RT-PCR. The GAPDH-normalized concentrations of MMP-2 and MMP-9 cDNA derived from skeletal muscles of healthy controls (4 samples) and patients with acute limb ischemia (12 samples) or CLI (18 samples) are presented as means ± SD. * $p \leq 0.05$.

to both categories of PAD (acute limb ischemia and CLI) than in controls. In contrast, the MMP-2 mRNA/cDNA levels were much higher in samples from controls than in the samples of the acute limb ischemia and CLI patients. The cDNA/cRNA levels for minor basement membrane components [laminin, perlecan, entactin (i.e. nidogen), tenascin, syndecan (i.e. heparan sulfate proteoglycan 2), SPARC and fibronectin] were mostly higher in samples from acute limb ischemia and lower in samples from CLI patients than in samples from controls. Expression signals for other MMPs (MMP-3, MMP-7 and MMP-15) and minor collagens of the vascular basement membrane (collagen type XV and type XVIII) were not detected by the gene array analysis in any of the individuals/patients (data not shown).

The analysis of MMP cDNA expression was extended by performing qRT-PCR on skeletal muscle samples derived from control individuals (4 samples) and patients with acute limb ischemia (12 samples from 4 patients) and CLI (18 samples from 9 patients) exemplified for MMP-2 and MMP-9. As shown in figure 3, the cDNA concentration of MMP-2 in skeletal muscles of control individuals was similar to that in CLI patients and significantly lower in samples from acute limb ischemia patients. In contrast, MMP-9 cDNA levels were significantly higher in skeletal muscles of acute limb ischemia and CLI patients than in controls. The correlation coefficients for the cDNA concentrations in those 12 samples that had been subjected to both gene array analysis and qRT-PCR were 0.51 for MMP-2 and 0.87 for MMP-9.

If immunoreactivity for MMPs and TIMPs was detected in skeletal muscle samples from control patients, patients with acute limb ischemia or CLI patients, it was always present at identical cellular/subcellular sites. Thus, immunoreactivity patterns differed only quantitatively and not qualitatively between the patient cohorts (fig. 4). Immunoreactivity for MMP-2 was associated with capillary endothelial cells around skeletal muscle fibers and, less frequently, with larger vessel. In samples of CLI and acute limb ischemia patients, MMP-9 was present only around larger vessels, particularly within the tunica media of arteries. In skeletal muscles of control individuals, no specific immunoreactivity for this metalloproteinase was detected. Immunoreactivity for MMP-19 was localized only in larger vessels occurring within the smooth muscle cells of the tunica media and the endothelial layer. TIMP-1 was expressed inside small skeletal muscle fibers and in association with free cells, particularly those located close to the skeletal muscle capillaries. Immunohistochemistry on serial sections with anti-CD163 antibodies revealed that many of these TIMP-1-positive free cells (approximately 70%) represent late stage inflammatory macrophages. Collagen type IV immunoreactivity was strongly expressed in basement membranes around capillaries and weakly on the surface of the skeletal muscle fibers. Specific immunoreactivity for MMP-3 and TIMP-2 was not detected (data not shown).

Semiquantitative grading of immunoreactivity showed MMP-2, MMP-19 and TIMP-1 to be less abundant in skeletal muscles derived from control individuals than PAD patients (suffering from acute limb ischemia or

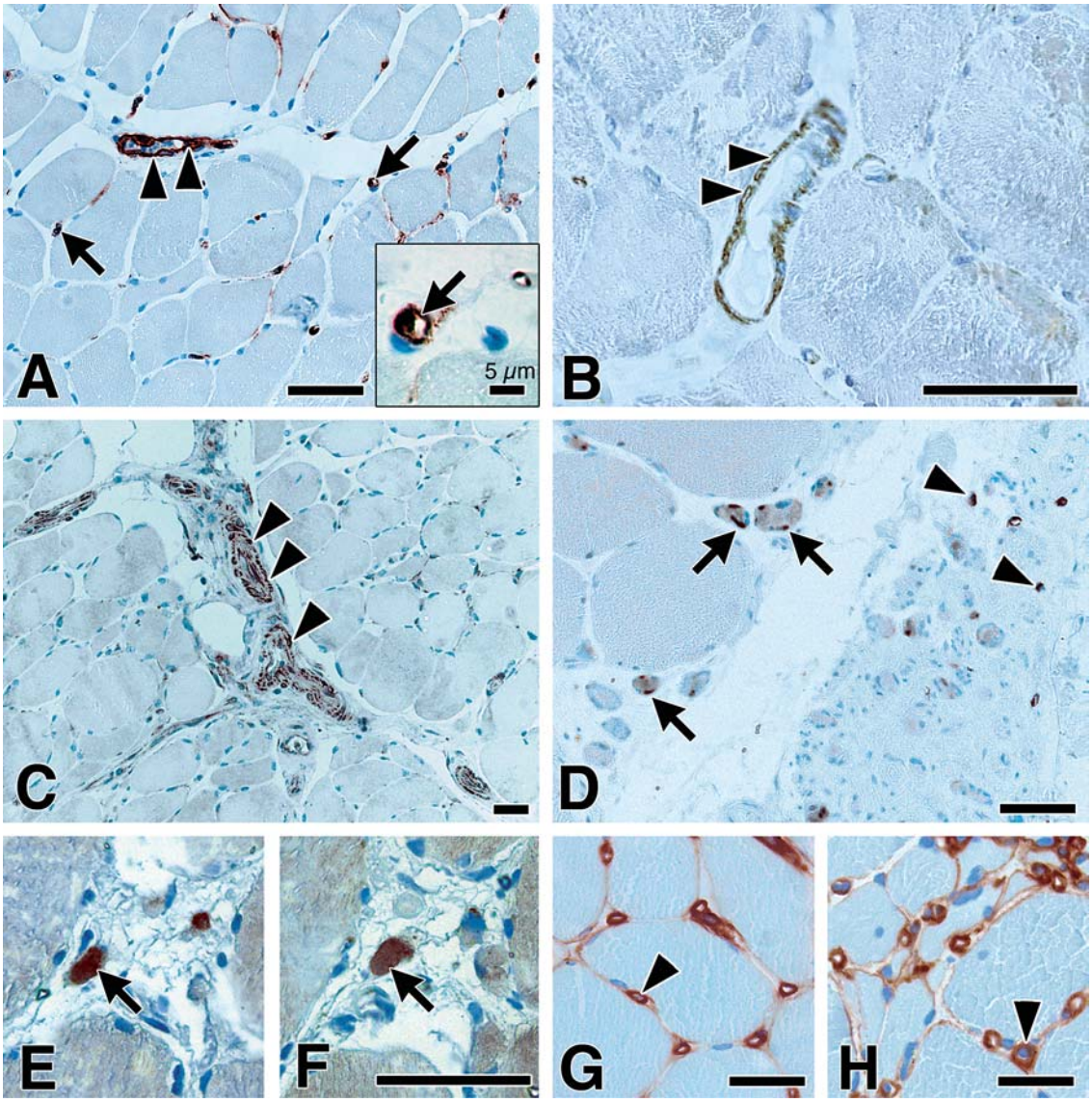

Fig. 4. Immunohistochemical analysis of skeletal muscle samples for MMP-2 (**A**), MMP-9 (**B**), MMP-19 (**C**), TIMP-1 (**D**, **E**), CD163 (**F**) and collagen type IV (**G**, **H**). In general, the localization of specific immunoreactivity for each protein did not differ in the 3 patient cohorts if immunostaining was observed. **A** Immunoreactivity for MMP-2 was detected in the tunica media of larger arteries (arrowheads) and around the microcirculation (arrows). The inset reveals MMP-2 to be located within the capillary endothelial cells. Sample: distal gastrocnemius muscle of acute limb ischemia patient SE **B** Immunoreactivity for MMP-9 was present in the tunica media of some larger vessels apparently only in association with smooth muscle cells (arrowheads). Sample: proximal tibialis anterior muscle of CLI patient BE **C** Immunoreactivity for MMP-19 occurred within the smooth muscle cells of the tunica media (arrowheads) and occasionally within the endothelial cells of larger vessels. Sample: distal gastrocnemius muscle of CLI patient FB **D** Immunoreactivity for TIMP-1 was detected within free cells located between degenerated skeletal muscle fibers (arrowheads) often in close contact with the capillaries and occasionally clustered in small muscle fibers (arrows). Sample: distal gastrocnemius muscle of acute limb ischemia patient BW **E**, **F** Many free cells (arrows) immunoreactive for TIMP-1 (**E**) also expressed CD168 (**F**), suggesting that they represent late stage inflammatory macrophages. Sample: distal gastrocnemius muscle of acute limb ischemia patient SE **G**, **H** Collagen type IV immunoreactivity was detectable in high quantities in pericapillary basement membranes (arrowheads) and weakly on the surface of the skeletal muscle fibers of control patients [proximal tibialis anterior muscle of patient GM (**G**)] and PAD patients [proximal tibialis anterior muscle of CLI patient BE (**H**)]. All sections were counterstained to visualize the cell nuclei. Scale bars = 50 μm.

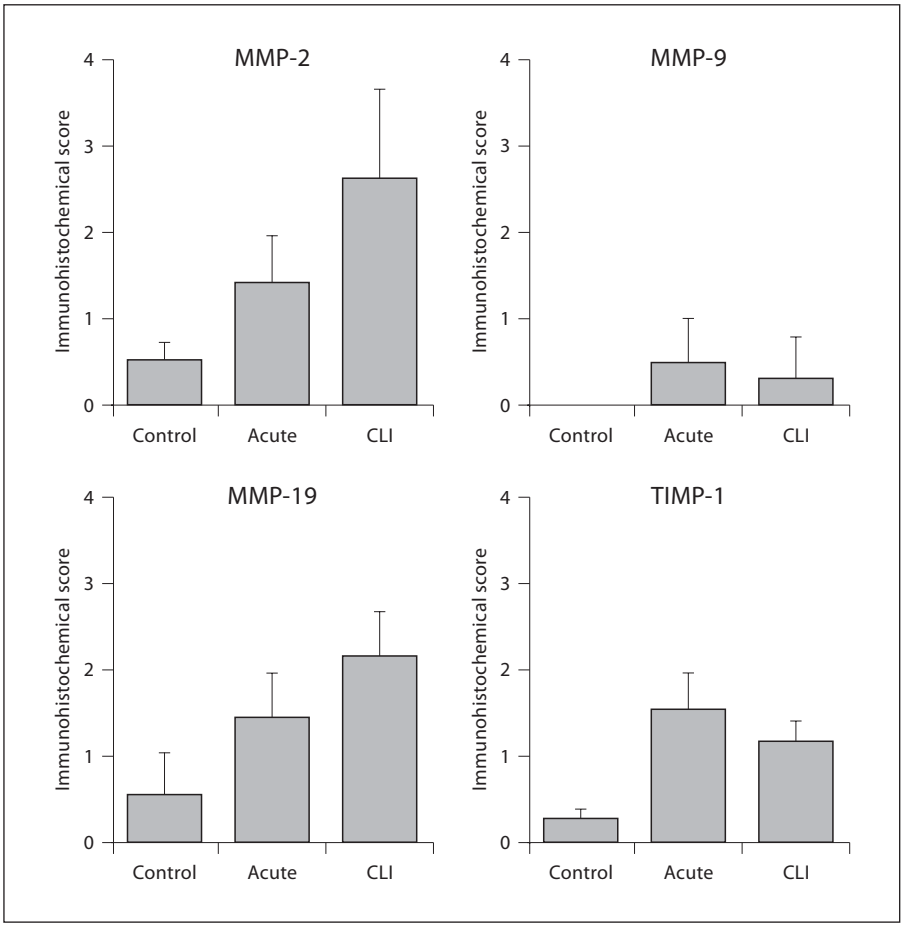

Fig. 5. Summary of the semiquantitative immunohistochemical analysis. Using a 5-step scale, immunoreactivity for MMP-2, MMP-9, MMP-19 and TIMP-1 was graded on skeletal muscle sections derived from healthy controls (3 samples) and patients with acute limb ischemia (10 samples) or CLI (18 samples). Means ± SD are represented.

CLI). Correspondingly, MMP-9 was observed only in samples from PAD patients, but not from controls (fig. 5). Because the extent of the collagen type IV immunoreactivity varied only slightly on the light microscopy level, we were not able to assess with certainty its thickness and consequently renounced to perform a semiquantitative analysis.

Discussion

The present study was performed to compare the morphology of skeletal muscle capillaries in PAD patients with CLI or acute limb ischemia and healthy control patients. Furthermore, the expression patterns of genes known to be involved in the remodeling of the vascular basement membrane were determined in skeletal muscle samples derived from these 3 patient cohorts. At the cDNA level, quantitative data for collagen type IV, MMPs and TIMPs were obtained by a gene array analysis, and the results for MMP-2 and MMP-9 were confirmed by qRT-PCR. A semiquantitative immunohistochemical analysis for MMP-2, MMP-9, MMP-19 and TIMP-1 was undertaken to ascertain whether the registered changes in mRNA levels were reflected in protein biosynthesis. A similar gene array analysis was undertaken to determine the pathways of angiogenesis factor in skeletal muscle of CLI patients and patients suffering from acute limb ischemia in comparison to control individuals [17].

Although not morphometrically quantified in our investigation, the basement membrane around skeletal muscle capillaries was regularly thicker in CLI patients than in asymptotic control patients, while it was slightly increased in acute limb ischemia patients. In an earlier study [5], we correlated the thickness of the microvascular basement membrane in skeletal muscle of CLI patients with cardiovascular risk factors. The statistical analysis revealed that its thickness is not related to hypertension, hypercholesterolemia, smoking and age but slightly to diabetes. These data are in accordance with the results of

the study by Feingold et al. [18] that correlated the thickening of the basement membrane around skeletal muscle capillaries in humans with diabetes but not with age. Williamson et al. [19] found a 25% thicker basement membrane around capillary basement membranes in skeletal muscle of 64 ± 3-year-old individuals in comparison to 24 ± 3-year-old individuals, but concluded with regard to additional experiments that physical inactivity rather than aging per se is responsible for muscle capillary basement thickening with advancing age. Thus, the development of an enlarged basement membrane is apparently not a physiological process occurring naturally during aging.

Collagens (mainly collagen type IV) as the major components comprise more than 50% of all proteins expressed in the basement membrane [review in 6]. The cDNA levels of collagen type IV chains A1 and A2, that are the dominant chains in skeletal muscle [7], were higher expressed in skeletal muscles of patients with acute limb ischemia than in those of CLI patients and control individuals. The minor components of the basement membrane showed similarly changed levels in the comparison of the patient cohorts. These data indicate that the expression of genes involved in basement membrane remodeling is upregulated at the mRNA level in response to acute tissue ischemia, whereas slowly progressive ischemia is not accompanied by an enhanced transcription of mRNA for collagen type IV.

Hitherto, ischemia-induced changes in the regulation of collagen type IV have not been reported in humans. However, the collagen type I protein has been shown to be strongly upregulated in the skin of CLI patients [20]. In accordance with our data on humans, the transcription of collagen type IV was induced in response to acute ischemia in skeletal muscle of mice [21] whilst the protein itself was more prone to degradation after ischemia/reperfusion in rats [22]. Furthermore, a hypoxia-induced augmentation in the biosynthesis of collagen type IV was observed also in the rat heart [23].

Although present at low levels, MMP-9 was clearly upregulated in CLI patients compared to controls according to the gene array, qRT-PCR and semiquantitative immunohistochemical analyses. Hence, MMP-9 expression is induced both at mRNA as well as protein levels in these patients. These results are in accordance with the observation that circulating levels of MMP-9 correlate positively with the severity of CLI [24, 25]. Correspondingly, the serum levels of MMP-9 correlate positively with the progression and fatal outcome of coronary artery disease [26].

Compared with controls, patients with CLI or acute limb ischemia contained higher levels of MMP-19, both at the mRNA and protein levels. To the best of our knowledge, this is the first report of MMP-19 expression in skeletal muscle. However, the expression pattern and regulation of MMP-19 described here are consistent with the results of a study in which MMP-19 was found to be expressed in vascular smooth muscle cells in a patient suffering from rheumatoid arthritis, and to be upregulated during acute and chronic inflammation [27].

Our gene array analysis revealed the cDNA concentrations of TIMP-1 to be higher in skeletal muscles of patients with acute limb ischemia than in controls and CLI patients. Likewise, at the protein level immunoreactivity for TIMP-1 was more abundant in muscle samples of acute limb ischemia patients than in control individuals. Patients with CLI held an intermediate position in the immunohistochemical analysis. These results indicate that in CLI patients, TIMP-1 is elevated above the control value at the protein but not at the mRNA level. The upregulation of TIMP-1 at the protein level could account for its merit as a serum marker for CLI [24]. However, an increase in TIMP-1 availability can be associated with the development of many other vascular remodeling processes, e.g. during atherosclerosis and restenosis [8, 28].

The cDNA levels of MMP-2 were lower in CLI patients than in controls, whereas immunoreactivity for the protein was higher. These findings indicate that less mRNA, but more protein, is expressed in skeletal muscles of CLI patients than healthy control individuals.

The apparent discrepancy in the regulation of TIMP-1 and MMP-2 expression at the cDNA (mRNA) and protein levels might be of biological relevance. A decrease in the control mRNA levels could represent a 'switching off' signal at times when the protein concentration is still high. This observation highlights the need to supplement mRNA-based methods with protein data, especially when evaluating integrated systems in vivo.

The data of qRT-PCR were normalized to GAPDH as reference gene because a statistical analysis revealed that this gene represents a reliable housekeeping gene in our samples [Ganster et al., unpubl. data]. We assume that the similar fiber type composition of the skeletal muscles used in our investigation (tibialis anterior, gastrocnemius caput lateralis) goes along with similar GAPDH levels. However, other studies showed that GAPDH levels might be regulated, e.g. during tissue injury [29] or resistance exercise [30]. Thus, part of the discrepancy in the expression between gene array analysis/qRT-PCR and semi-

quantitative immunohistochemistry may be due to normalization with GAPDH.

Immunohistochemistry revealed MMP-9 and MMP-19 to be associated exclusively with the tunica media of larger vessels, whereas MMP-2 was also located around the capillaries. These findings accord with existing data. MMP-2, MMP-9 and MMP-19 are known to be present in vascular smooth muscle vessels of larger vessels in many organs, including skeletal muscle [9, 27]. The expression of MMP-2 in skeletal muscle capillaries has also been reported before [31]. The occurrence, but not the localization, of TIMP-1 in skeletal muscle has been documented hitherto. Our study revealed this protein to be associated with smaller muscle fibers and macrophages that accumulate in the extracellular matrix of degenerating and regenerating skeletal muscle fibers [15].

Taken together, our molecular and immunohistochemical data indicate that in patients suffering from acute limb ischemia the rate of biosynthesis of collagen type IV, collagen-degrading MMPs and TIMP-1 is upregulated in skeletal muscle. Since only narrow cuffs of basement membrane were detected around the microcirculatory vessels of patients with acute limb ischemia, we assume that the assembly and degradation of this entity is well balanced in this patient cohort. However, it is also conceivable that the mRNA induction of collagen type IV is sustained for an insufficient period to support the formation of a thickened vascular basement membrane that is so characteristic for the skeletal muscles of CLI patients [5]. To finally determine the contribution of MMPs to this CLI-associated basement membrane thickening, additional assays measuring their proteolytic activity (e.g. zymograms) should be performed.

Although the transcription of collagen type IV was not induced, and the expression of capillary-specific MMP-2 was markedly upregulated at the protein level in CLI patients, we postulate that basement membrane synthesis and MMP expression are unbalanced in the skeletal muscle capillaries of these patients. This situation might be accounted for by the increased production of the MMP-2-specific inhibitor TIMP-1 by macrophages. Such inflammatory cells might be attracted by the continuously activated endothelium [32] as it is has been observed in the skeletal muscle capillaries of CLI patients [5]. In larger vessels, MMP-2, MMP-9 and MMP-19 were present at high concentrations in the absence of TIMPs. Hence these larger vessels might be protected from an enlargement of the basement membrane, which indeed was never observed.

To the best of our knowledge, a thickening of the vascular basement membrane in skeletal muscle occurring in response to hypoxia has been observed only in one animal model [33]. However, this previous study yielded no information with respect to the molecular mechanisms underlying the basement membrane remodeling. It seems reasonable to suppose that nitric oxide is important in this process since this molecule plays a pivotal role in the recruitment of smooth muscle cell actin-immunoreactive cells [34]. Owing to the variability in CLI patients (such as distribution and grade of muscle degeneration), the samples from the affected patient population are too heterogeneous for a systematic analysis of all molecular mechanisms underlying the basement membrane thickening. This must await the development of new methods for experimentally inducing ischemia in animals, for example, by consecutive double ligation [35].

The mean age of the 3 patient cohorts varied, albeit these differences were not statistically significant. In general, the analysis of human samples often goes along with methodological and biological limits, e.g. it is not easy to have access to perfectly age-matched patient cohorts or to collect a sufficient number of samples. Furthermore, samples from CLI patients are often very heterogeneous as mentioned above. Despite these drawbacks it seems necessary to perform investigations on human samples that reflect the phenotype associated with the development of a pathological disease and thus give insight into the clinical situation.

In summary, our experimental data reveal the basement membrane around vessels of the microcirculation to be enlarged in patients with longstanding CLI, even though the expression of several MMPs is elevated above normal levels. At present, it is unclear whether the demonstrated changes in gene expression are related to hypoxia or the inflammation associated with the progression of the atherosclerotic disease. Nevertheless, the ultrastructure of the vascular basement membrane is undoubtedly disturbed in the skeletal muscles of PAD patients. This finding could be relevant in the presentation and therapeutic consequences of limb ischemia.

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References

1 Meru AV, Mittra S, Thyagarajan B, Chugh A: Intermittent claudication: an overview. Atherosclerosis 2006;187:221–237.
2 Baumgartner I, Schainfeld R, Graziani L: Management of peripheral vascular disease. Annu Rev Med 2005;56:249–272.
3 Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y: Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med 2003;9:604–613.
4 Tang GL, Chang DS, Sarkar R, Wang R, Messina LM: The effect of gradual or acute arterial occlusion on skeletal muscle blood flow, arteriogenesis, and inflammation in rat hindlimb ischemia. J Vasc Surg 2005;41:312–320.
5 Baum O, Djonov V, Ganster M, Widmer M, Baumgartner I: Arteriolization of capillaries and FGF-2 upregulation in skeletal muscles of patients with chronic peripheral arterial disease. Microcirculation 2005;12:527–537.
6 Kalluri R: Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 2003;3:422–433.
7 Kovanen V: Intramuscular extracellular matrix: complex environment of muscle cells. Exerc Sport Sci Rev 2002;30:20–25.
8 Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827–839.
9 Galis ZS, Khatri JJ: Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 2002;90:251–262.
10 Brown MD, Hudlicka O: Modulation of physiological angiogenesis in skeletal muscle by mechanical forces: involvement of VEGF and metalloproteinases. Angiogenesis 2003;6:1–14.
11 Carmeli E, Moas M, Reznick AZ, Coleman R: Matrix metalloproteinases and skeletal muscle: a brief review. Muscle Nerve 2004;29:191–197.
12 Second European consensus document on chronic critical leg ischemia. Circulation 1991;84(suppl IV):IV1–IV26.
13 Gehlenborg N, Dietzsch J, Nieselt K: A framework for visualization of microarray data and integrated meta information. Inf Vis 2005;4:164–175.
14 Sadowski T, Dietrich S, Muller M, Havlickova B, Schunck M, Proksch E, Muller MS, Sedlacek R: Matrix metalloproteinase-19 expression in normal and diseased skin: dysregulation by epidermal proliferation. J Invest Dermatol 2003;121:989–996.
15 McLennan IS: Degenerating and regenerating skeletal muscles contain several subpopulations of macrophages with distinct spatial and temporal distributions. J Anat 1996;188:17–28.
16 Breitling R, Armengaud P, Amtmann A, Herzyk P: Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett 2004;573:83–92.
17 Tuomisto TT, Rissanen TT, Vajanto I, Korkeela A, Rutanen J, Yla-Herttuala S: HIF-VEGF-VEGFR-2, TNF-α and IGF pathways are upregulated in critical human skeletal muscle ischemia as studied with DNA array. Atherosclerosis 2004;174:111–120.
18 Feingold KR, Browner WS, Siperstein MD: Prospective studies of muscle capillary basement membrane width in prediabetics. J Clin Endocrinol Metab 1989;69:784–789.
19 Williamson JR, Hoffmann PL, Kohrt WM, Spina RJ, Coggan AR, Holloszy O: Endurance exercise training decreases capillary basement membrane width in older nondiabetic and diabetic adults. J Appl Physiol 1996;80:747–753.
20 Dalton SJ, Mitchell DC, Whiting CV, Tarlton JF: Abnormal extracellular matrix metabolism in chronically ischemic skin: a mechanism for dermal failure in leg ulcers. J Invest Dermatol 2005;125:373–379.
21 Lee CW, Stabile E, Kinnaird T, Shou M, Devaney JM, Epstein SE, Burnett MS: Temporal patterns of gene expression after acute hindlimb ischemia in mice: insights into the genomic program for collateral vessel development. J Am Coll Cardiol 2004;43:474–482.
22 Roach DM, Fitridge RA, Laws PE, Millard SH, Varelias A, Cowled PA: Up-regulation of MMP-2 and MMP-9 leads to degradation of type IV collagen during skeletal muscle reperfusion injury; protection by the MMP inhibitor, doxycycline. Eur J Vasc Endovasc Surg 2002;23:260–269.
23 Perhonen M, Wang W, Han X, Ruskoaho H, Takala TE: Right ventricular collagen type III and IV gene expression increases during early phases of endurance training in hypobaric hypoxic condition. Basic Res Cardiol 1997;92:299–309.
24 Tayebjee MH, Tan KT, MacFadyen RJ, Lip GY: Abnormal circulating levels of metalloprotease 9 and its tissue inhibitor 1 in angiographically proven peripheral arterial disease: relationship to disease severity. J Intern Med 2005;257:110–116.
25 Signorelli SS, Malaponte G, Libra M, Di Pino L, Celotta G, Bevelacqua V, Petrina M, Nicotra GS, Indelicato M, Navolanic PM, Pennisi G, Mazzarino MC: Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease. Vasc Med 2005;10:1–6.
26 Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, Meyer J, Cambien F, Tiret L: Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation 2003;107:1579–1585.
27 Kolb C, Mauch S, Krawinkel U, Sedlacek R: Matrix metalloproteinase-19 in capillary endothelial cells: expression in acutely, but not in chronically, inflamed synovium. Exp Cell Res 1999;250:122–130.
28 Puhakka HL, Turunen P, Rutanen J, Hiltunen MO, Turunen MP, Yla-Herttuala S: Tissue inhibitor of metalloproteinase 1 adenoviral gene therapy alone is equally effective in reducing restenosis as combination gene therapy in a rabbit restenosis model. J Vasc Res 2005;42:361–367.
29 Bustin SA: Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29:23–39.
30 Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, Tarnopolsky MA: Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. Physiol Genomics 2004;18:226–231.
31 Milkiewicz M, Haas TL: Effect of mechanical stretch on HIF-1α and MMP-2 expression in capillaries isolated from overloaded skeletal muscles: laser capture microdissection study. Am J Physiol Heart Circ Physiol 2005;289:H1315–H1320.
32 Rajashekhar G, Willuweit A, Patterson CE, Sun P, Hilbig A, Breier G, Helisch A, Clauss M: Continuous endothelial cell activation increases angiogenesis: evidence for the direct role of endothelium linking angiogenesis and inflammation. J Vasc Res 2006;43:193–204.
33 Hoppeler H, Hudlicka O, Uhlmann E, Claassen H: Capillaries and mitochondria in rat muscles with limited blood supply: a report on a morphometric study in progress; in Okyayuz-Bakluti I, Hudlicka O (eds): Muscle Ischaemia: Functional and Metabolic Aspects. Munich, Wolf und Sohn, 1988, pp 89–98.
34 Yu J, deMuinck ED, Zhuang Z, Drinane M, Kauser K, Rubanyi GM, Qian HS, Murata T, Escalante B, Sessa WC: Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. Proc Natl Acad Sci USA 2005;102:10999–11004.
35 Brown MD, Kelsall CJ, Milkiewicz M, Anderson S, Hudlicka O: A new model of peripheral arterial disease: sustained impairment of nutritive microcirculation and its recovery by chronic electrical stimulation. Microcirculation 2005;12:373–381.