

# Single and combined effects of $\alpha v\beta 3$ - and $\alpha 5\beta 1$ -integrins on capillary tube formation in a human fibrinous matrix

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**Abstract** The fibrinous exudate of a wound or tumor stroma facilitates angiogenesis. We studied the involvement of RGD-binding integrins during tube formation in human plasma-derived fibrin clots and human purified fibrin matrices. Capillary-like tube formation by human microvascular endothelial cells in a 3D plasma-derived fibrinous matrix was induced by FGF-2 and TNF- $\alpha$  and depended largely on cell-bound u-PA and plasmin activities. While tube formation was minimally affected by the addition of either the  $\alpha v\beta 3$ -integrin inhibiting mAb LM609 or the  $\alpha 5$ -integrin inhibiting mAb IIA1, the general RGD-antagonist echistatin completely inhibited this process. Remarkably, when  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrins were inhibited simultaneously, tube formation was reduced by 78%. It was accompanied by a 44% reduction of u-PA antigen accumulation and 41% less production of fibrin degradation products.  $\alpha v\beta 5$ -integrin-blocking antibodies further enhanced the inhibition by mAb LM609 and mAb IIA1 to 94%, but had no effect by themselves.  $\alpha v$ -specific cRGD only inhibited angiogenesis when  $\alpha 5\beta 1$ -integrin was simultaneously blocked. Endostatin mimicked the effect of  $\alpha 5\beta 1$ -integrin and inhibited tube formation only in the presence of LM609 or cRGD (73 and 80%, respectively). Comparable results were obtained when purified fibrin

matrices were used instead of the plasma-derived fibrinous matrices. These data show that blocking of tube formation in a fibrinous exudate requires the simultaneous inhibition of  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrins. This may bear impact on attempts to influence angiogenesis in a fibrinous environment.

**Keywords** Angiogenesis ·  $\alpha v\beta 3$ -integrin ·  $\alpha 5\beta 1$ -integrin · LM609 · Endostatin

## Introduction

Angiogenesis, the outgrowth of new microvascular structures from the preexisting vasculature, is pivotal for embryonic development [1]. Once the organism has become adult, angiogenesis is limited to the endometrium and ovary. However, as part of normal or deranged tissue-repair processes, it is induced after wounding and in a number of diseases, such as cancer, diabetic retinopathy, and rheumatoid arthritis [1, 2]. Repair-associated angiogenesis is normally accompanied by fibrin deposition and accumulation of cytokine-producing leukocytes [3–6]. The leakiness of tumor vessels and the presence of tissue factor in the interstitial tissue favor the deposition of a fibrinous matrix as part of the tumor stroma. Such a fibrinous matrix provides excellent scaffolding for the invasion of new vascular structures [4, 7, 8]. Thus, the temporary fibrinous scaffolding not only plays an important role in wound closure, but also supports cell invasion and angiogenesis in tumor stroma and in inflamed tissues.

The induction and activity of cell-bound proteolytic activities and matrix-binding receptors largely regulate the migration and invasion of endothelial cells during angiogenesis, in addition to structural epitopes in the extracellular or fibrinous matrix itself [9]. Both the cell-bound urokinase

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and plasmin activities and matrix metalloproteinases (MMPs), in particular membrane-type MMPs (MT-MMPs), play a pivotal role in migration and invasion of endothelial cells during the formation of tubular structures in a fibrin matrix [9–14].

Besides proteolysis, the formation of new adhesion sites between the invading cells and the matrix is required. Among the various types of adhesion molecules, the integrins particularly play an important role in tissue repair and tumor-induced angiogenesis [15–18]. Integrins are transmembrane glycoproteins consisting of noncovalently linked  $\alpha$ - and  $\beta$ -subunits, which can form various heterodimers [18, 19]. Presently, nine integrins have been recognized on endothelial cells [19]. The RGD-recognizing integrins  $\alpha v\beta 3$ -,  $\alpha v\beta 5$ -, and  $\alpha 5\beta 1$  facilitate cell binding to fibrin [20] and are expressed on the endothelial cells of newly formed vessels in a fibrinous matrix of wounds and tumors [21–23]. Quantitative alterations in  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrin expression in particular, modulate the adhesive and migratory properties of endothelial cells during angiogenesis in wound repair and tumor growth [24, 25]. This can be induced by provisional matrix molecules in the wound clot, such as fibrin, and local conditions, such as hypoxia [26, 27]. On the basis of the inhibitory activities of  $\alpha v$ - and  $\alpha v\beta 3$ -integrin antagonists, such as cRGD peptides and antibody LM609, on angiogenesis in several pathological conditions in animals it was postulated that such antagonist may be good candidates for inhibiting tumor angiogenesis [21]. However, this concept was challenged by the finding of Reynolds et al. [28, 29] that mice lacking  $\beta 3$ -integrins or both  $\beta 3$ - and  $\beta 5$ -integrins support tumor angiogenesis and tumor growth. This data indicates that the role of  $\alpha v$ -integrins in angiogenesis is more complex than originally thought [30–32]. In addition to  $\alpha v\beta 3$  inhibition, blocking of  $\alpha 5\beta 1$  can contribute to the regulation of angiogenesis [23]. The  $\alpha 5\beta 1$ -integrin and other integrins have been shown to be targets for endostatin, the C-terminal fragment of collagen XVIII [33, 34] which can strongly suppress tumor-induced angiogenesis in animals [35–37].

The formation of capillary-like structures in a fibrinous exudate can be mimicked by human microvascular endothelial cells (hMVECs) in a 3D-fibrin matrix [11]. With this model we reported previously that inhibition of  $\alpha v\beta 3$ -integrin activity by LM609 did not reduce the formation of capillary-like structures by hMVECs in a fibrin matrix [27]. Subsequently we observed that inhibition of RGD-binding integrins by echistatin prevented tube formation. Because both  $\alpha 5\beta 1$ - and  $\alpha v$ -integrins are involved in the binding of endothelial cells to a fibrinous matrix and may influence their activities mutually, we further evaluated how various RGD-binding integrins cooperate in the formation of capillary-like structures. This was studied both in a fibrin

matrix and in 3D-matrices consisting of a plasma clot to mimic the fibrinous exudate more closely. The involvement of RGD-binding integrins during neovascularization could also be demonstrated by tube formation in the murine fibrinous exudate.

## Materials and methods

### Materials

Cell culture reagents were purchased as previously described [38]. Human plasma and serum were obtained from a local blood bank, and plasma clot matrices were prepared from freshly obtained blood from 10 to 20 healthy donors. FGF-2 was purchased from Pepro Tech EC (London, UK) and human recombinant TNF- $\alpha$  from Sigma-Aldrich Chemie (Steinheim, Germany). Factor XIII was generously provided by Dr. H. Metzner and Dr. G. Seeman (Aventis Behring GmbH, Marburg, Germany). Thrombin was purchased from Leo Pharmaceutic Products (Weesp, The Netherlands). The active  $\alpha v\beta 3$ -blocking mAb LM609 and the  $\alpha v\beta 5$ -blocking mAb P1F6 were purchased from Chemicon (Temecula, CA). The  $\alpha 5$ -integrin blocking mAb IIA1 was obtained from Pharmingen (Hamburg, Germany). The  $\alpha 1$ -integrin blocking mAb 5E8D9 was obtained from Upstate (Milton Keynes, UK). Endostatin was produced as described by Yamaguchi et al. [36]. The biotinylated horse anti-mouse IgG was obtained from Vector Laboratories (Burlingame, CA) and the biotinylated donkey anti-rabbit was purchased from Amersham Pharmacia Biotech UK (Buckinghamshire, England). Rabbit anti- $\alpha 5$  and - $\beta 5$  integrins, both against the cytoplasmic domain of the integrin, were kindly provided by Dr. Guido Tarone (University of Turin, Italy). The blocking anti- $\beta 1$  mAb P4C10 was obtained from Millipore (Billerica, MA). The inhibitory anti-uPAR mAb H-2 was kindly donated by Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany). The human CD31 antigen, a monoclonal antibody, was from Novocastra Laboratories, Newcastle, UK. Pepsin was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Biotinylated thyramides were obtained from the Department of Endocrinology from the Leiden University Medical Center (The Netherlands). The horseradish peroxidase (HRP) Avidin Biotinylated Complex was purchased from DAKO (Glostrup, Denmark) and Novared from Vector Laboratories (Burlingame, CA). Time specimens of recanalized mural thrombi were kindly provided by Dr. H.W. Niessen (VU University Medical Center Amsterdam, Department of Pathology, Amsterdam, The Netherlands). cRGD, an  $\alpha v$ -inhibiting peptide and GRGDSP, an RGD-inhibiting peptide were obtained from Bachem (St. Helens, UK).

## Cell culture

Human foreskin microvascular cells (hMVECs) were isolated, cultured and characterized as previously described [39, 40].

## Immunohistochemical analysis

Tissue sections of a recanalized human mural thrombus were dewaxed by immersion in xylene and rehydrated in decreasing concentrations of ethanol. For HPS staining, the sections were counterstained with Mayer's hematoxylin, phloxin and saffron. After dehydration in a reversed ethanol–xylene series, the sections were prepared for microscopy.

Inhibition for endogenous peroxidase was accomplished by immersion in 1% hydrogen peroxidase in absolute methanol for 20 min at room temperature. The sections were washed in deionized water and equilibrated in PBS. Antigen retrieval for sections treated with anti-CD31 was successful when the sections were incubated in 0.1 mol/l sodium-citrate in a microwave at 700 W until boiling point was reached, followed by a period of 10 min at 180 W. In the case of LM609 and the rabbit anti-human antibody against the  $\alpha 5$ -integrin, appropriate antigen retrieval was reached when the sections were treated with 2 mg/ml pepsin for 75 min at room temperature. For sections treated with the rabbit anti-human antibody against the  $\beta 5$ -integrin, appropriate antigen retrieval was reached after a 10-min incubation at room temperature with proteinase K. Subsequently, all the sections were blocked by 5% BSA in PBS to prevent nonspecific binding. Incubation with the anti-CD31 (1:100), LM609 (10  $\mu$ g/ml), anti- $\alpha 5$  (whole rabbit serum diluted 1:400), and anti- $\beta 5$  (whole rabbit serum diluted 1:50) was done overnight at 4°C. After washes with PBS, the sections were exposed to the second antibody, the biotinylated horse anti-mouse IgG (1:400) or the donkey anti-rabbit (1:200) diluted in 1% BSA/PBS, for 1 h at room temperature. Afterwards, the sections were washed with PBS and incubated for 30 min at room temperature with the HRP Avidin Biotinylated Complex (ABCComplex). In order to amplify the signal of the sections stained with the anti- $\beta 5$ -integrin, biotinylated thyramides were added for 10 min at room temperature, followed again by the HRP ABCComplex for 30 min at room temperature. The sections were washed with PBS and stained with Novared for a period of 5–10 min. Finally, the sections were washed in aquadest, counterstained with hematoxylin, washed in running water and dehydrated in a reversed ethanol–xylene series, and prepared for microscopy.

## Preparation of the three-dimensional plasma and fibrin clots

Coagulation of plasma clots was performed as described by Engelse et al. [41]. Briefly, 2.5 U/ml Factor XIII and 1 U/ml thrombin were added to human plasma, and 300  $\mu$ l aliquots of this mixture was added to the wells of a 48-well plate. After clotting at room temperature for at least 30 min, the matrices were equilibrated at 37°C, 5% CO<sub>2</sub>/95% air atmosphere with serum-containing M199 to inactivate the thrombin. During the next 24 h, the matrices were washed three times with M199 to eliminate residual citrate. Fibrin matrices were prepared as previously described [11].

## In vitro tube formation assay

The formation of capillary-like tubes in plasma-derived fibrinous matrices was evaluated essentially as previously described in fibrin matrices [11]. Confluent hMVECs were seeded on top of the matrices. After seeding 24 h, the cells were stimulated with serum-containing M199 with or without (control) the presence of FGF-2 and TNF- $\alpha$  and the reagents to be tested. Every second day, the medium was removed and replaced by fresh stimulation medium and after 7 days, the formation of tubular structures was analyzed by phase contrast microscopy as previously described [11].

## ELISAs

u-PA antigen determinations as well as fibrin degradation products (FDP) determinations were performed by the commercially available immunoassay kits: u-PA EIA HS (Taurus, Leiden, The Netherlands) and Fibrinostika<sup>®</sup> FDP ELISA (Organon-Technika, Turnhout, Belgium). The monoclonal antibodies that are used in this uPA ELISA were made in our department and recognize all forms of uPA: latent (single-chain) uPA, active (two-chain) uPA, and u-PA complexed with PAI-1 in a similar manner [11, 42].

## Statistical analysis

Experiments were performed in duplicate and the results were expressed as mean  $\pm$  SEM in relation to the FGF-2/TNF- $\alpha$  condition. For statistical analysis, we used one-way ANOVA followed by the Dunnett's test as post-test. The Dunnett's test is a modified t-test that takes into account multiple comparisons with a control condition. Statistical significance was accepted at  $P < 0.05$ .

## Results

### Endothelial cells in a fibrinous matrix express $\alpha\nu\beta3$ -, $\alpha\nu\beta5$ - and $\alpha5\beta1$ -integrins

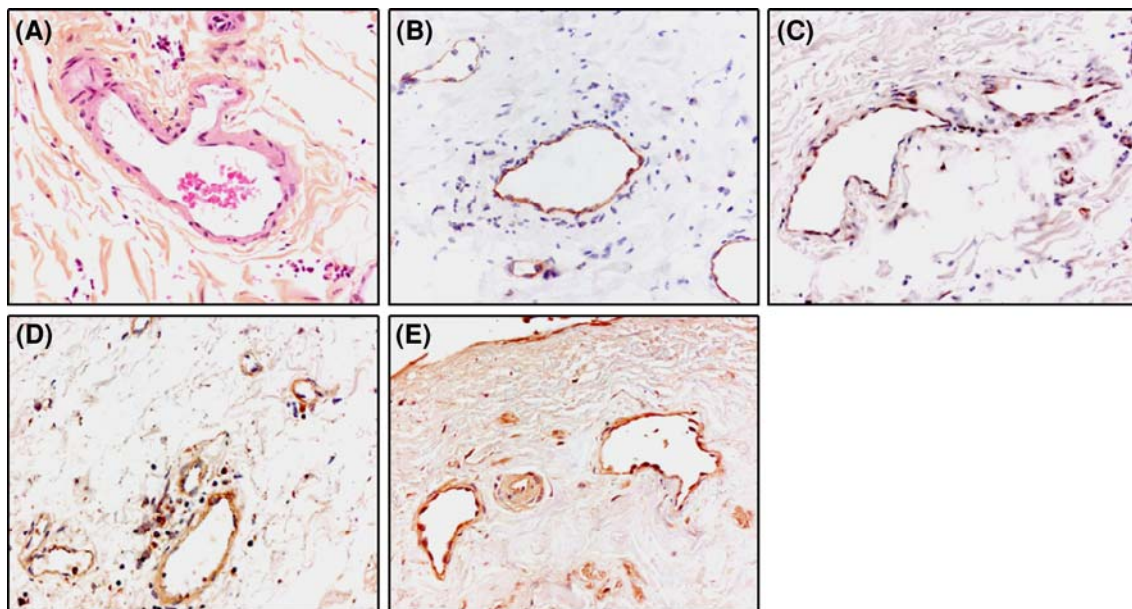
Endothelial cells produce several integrins, three of which have been reported to interact with fibrin:  $\alpha\nu\beta5$ -,  $\alpha\nu\beta3$ -, and  $\alpha5\beta1$ -integrins. To verify the presence of these integrins in new vascular structures formed in a fibrinous environment, immunohistochemical analysis of these integrins was performed in tissue sections of a recanalized human mural thrombus. As is shown in Fig. 1,  $\alpha\nu\beta3$ -integrin,  $\beta5$ -containing integrins reflecting  $\alpha\nu\beta5$ -integrin, and  $\alpha5$ -containing integrins, representing  $\alpha5\beta1$  integrin, were present in many vascular structures of these thrombi.

### Involvement of RGD-binding integrins in the formation of tubular structures in a 3D plasma-derived fibrinous matrix

To investigate the involvement of integrins in tube formation in a human fibrinous exudate, we used an in vitro matrix consisting of clotted plasma to study the formation of capillary-like tubes by human microvascular endothelial cells (hMVECs). For that, hMVECs were cultured on top of the plasma-derived fibrinous matrix, and capillary-like structures were formed after 4–6 days of culture in the continuous presence of both FGF-2 (10 ng/ml) and TNF- $\alpha$  (10 ng/ml), whereas addition of either FGF-2 or the

cytokine TNF- $\alpha$  hardly induced the formation of tubular structures comparable to previous observations on hMVEC-mediated tube formation in plasma-derived fibrinous matrices [41] and fibrin matrices made using purified fibrinogen [11].

When echistatin (1  $\mu$ g/ml), a general RGD inhibitor, was added simultaneously with FGF-2/TNF- $\alpha$  to hMVECs grown on a human plasma-derived fibrinous matrix, hardly any capillary-like structures were formed ( $18.6 \pm 4.7\%$  of the amount formed by FGF-2/TNF- $\alpha$ -stimulated cells,  $n = 3$ ,  $P < 0.001$ ). The nature of the RGD-binding integrins was further investigated using blocking mAbs against  $\alpha5\beta1$ -,  $\alpha\nu\beta3$ -, and  $\alpha\nu\beta5$ -integrins. Addition of either the  $\alpha\nu\beta3$ -integrin blocking mAb LM609, the  $\alpha\nu\beta5$ -integrin antagonist mAb P1F6, or the  $\alpha5$ -integrin inhibiting mAb IIA1 did not or only marginally affect the formation of tubular structures in a plasma clot (Fig. 2a). Their effectiveness was verified by their ability to inhibit cell binding to fibronectin by  $61 \pm 6\%$  (mAb IIA1) or vitronectin-coated dishes by  $55 \pm 7\%$  (mAb LM609) and  $40 \pm 14\%$  (mAb P1F6). Interestingly, when mAb IIA1 was added together with either LM609 or P1F6, the formation of capillary-like structures was significantly decreased. The combination of anti- $\alpha\nu\beta3$  (LM609) and anti- $\alpha5$  (IIA1) integrins was most effective. Addition of anti- $\alpha\nu\beta5$  P1F6 to the combination of mAb LM609 and mAb IIA1 resulted in a further reduction of the formation of capillary-like structures (Fig. 2a). Another mAb, 5E8D9, that blocks the  $\alpha1$ -integrin, did not affect tube formation neither by itself



**Fig. 1** Localization of RGD-binding integrins in endothelial cells in vivo. Immunohistochemistry was performed on paraffin sections of a recanalized mural thrombus formed in a human coronary artery as described in Materials and Methods. **a** Phloxin staining;

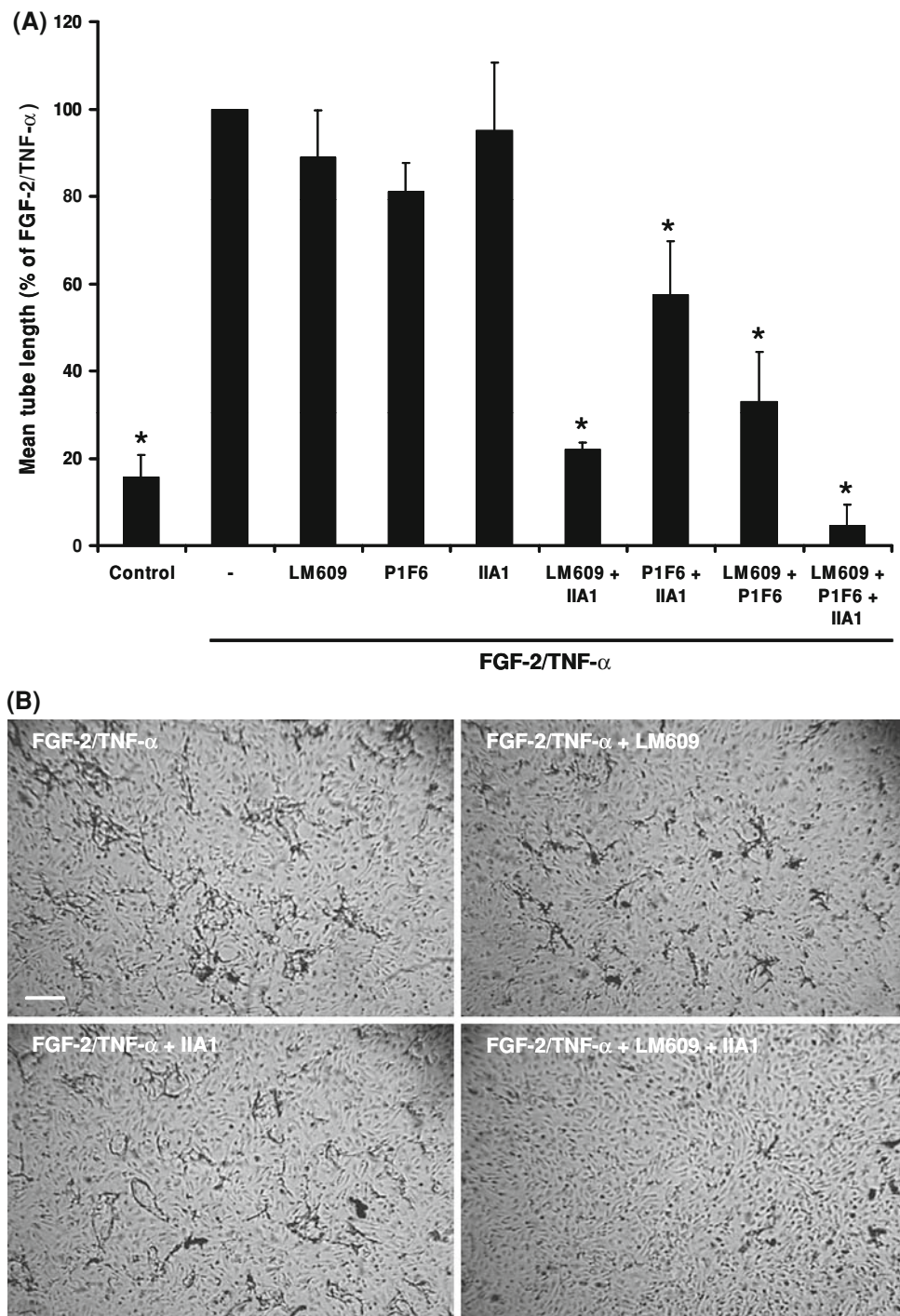
**b** Immunostaining of endothelial cells by CD31; **c** Immunostaining of  $\alpha\nu\beta3$ -integrin by mAb LM609; **d** Immunostaining of  $\alpha\nu\beta5$ -integrin by rabbit anti-human  $\beta5$ -integrin antibody; **e** Immunostaining of  $\alpha5\beta1$ -integrin by rabbit anti-human  $\alpha5$ -integrin antibody

nor in combination with mAb LM609 or mAb IIA1 ( $86 \pm 2$  and  $82 \pm 1$  of control, respectively). The  $\beta 1$ -integrin blocking mAb P4C10, which inhibits  $\alpha 5\beta 1$ - and  $\alpha 3\beta 1$ -integrins, had no effect on tube formation when added as a single antibody (data not shown).

The presence of either mAb LM609 or mAb IIA1 during the tube formation assay did not affect cell

attachment, once the cells had been seeded on top of a plasma-derived fibrinous matrix (compare the intact monolayer of cells in Fig. 2b). No apoptotic cells were observed in the presence of integrin inhibitors as determined by the absence of fragmentation of nuclei, after DAPI staining and evaluation by digital imaging microscopy (data not shown).

**Fig. 2** Blocking of  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrins results in a combined effect on the inhibition of capillary-like tube formation in plasma-derived fibrinous matrices. HMVECs cultured on a three-dimensional fibrinous plasma clot were not stimulated (control) or stimulated with FGF-2/TNF- $\alpha$  in the absence or presence of the  $\alpha v\beta 3$ -blocking mAb LM609 (10  $\mu\text{g}/\text{ml}$ ), the  $\alpha v\beta 5$ -blocking mAb P1F6 (10  $\mu\text{g}/\text{ml}$ ), the  $\alpha 5$ -blocking mAb IIA1 (2  $\mu\text{g}/\text{ml}$ ) or the combinations of these mAbs. After 6 days of culturing, the mean tube length of duplicate wells was quantified ( $\text{mm}/\text{cm}^2$ ) as described (a). The data represent mean  $\% \pm \text{SEM}$  of FGF-2/TNF- $\alpha$  stimulated tube formation of 3–7 independent experiments. The mean tube length of FGF-2/TNF- $\alpha$  in the plasma-derived fibrinous matrix was  $70 \pm 20 \text{ mm}/\text{cm}^2$ . **b** After 6 days of culturing, non-phase photomicrographs were taken of hMVECs stimulated with FGF-2/TNF- $\alpha$  in the absence or presence of LM609, IIA1 or the combination of these mAbs in the concentrations described above. Bar represents 300  $\mu\text{m}$ . \*  $P < 0.01$  as compared to 100% (=FGF-2/TNF- $\alpha$  stimulated condition)



### Simultaneous addition of mAb LM609 and mAb IIA1 affects u-PA and FDP production

The reduced ingrowth of capillary-like structures after exposure to mAb LM609 and mAb IIA1 was accompanied by a reduced accumulation of u-PA in the conditioned medium of the tube-forming cells (Table 1). Furthermore, the reduced formation of tubular structures was paralleled by a decreased generation of fibrin degradation products (Table 1). The individual antibodies hardly affected these parameters. This suggests that the simultaneous interference with  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrins results in a diminished u-PA accumulation in the supernatants of the FGF-2/TNF- $\alpha$ -treated cells and a diminished generation of FDP products. Experiments using the uPA receptor (uPAR) blocking antibody H-2 indicated that the differences in uPA were not due to decreased uPAR availability on the endothelial cells. The mAb H-2 displaces uPA from the uPAR and thereby prevents the uptake and degradation of uPA by the endothelial cells. In the presence of mAb H-2, the accumulation of uPA in the conditioned medium increased by 160–180% in all conditions as compared with each counterpart without H-2.

### Endostatin acts in concert with mAb LM609 and cRGD in inhibiting capillary-like tube formation

Endostatin, at a concentration of 10  $\mu\text{g/ml}$ , affected the FGF-2/TNF- $\alpha$ -induced formation of tubular structures by hMVECs in a plasma-derived fibrinous matrix minimally (Fig. 3). When endostatin was given simultaneously with mAb LM609, but not with mAb IIA1, the formation of tubular structures was largely inhibited (Fig. 3). Apparently, endostatin was able to mimic the effect of a  $\alpha 5$ -integrin blocking e.g., mAb IIA1 (compare Fig. 3 and 2a).

The interaction of  $\alpha 5\beta 1$ - and  $\alpha v\beta 3$ -integrins was further underscored using cyclic RGD peptides with specificity for

$\alpha v$ -integrins [43]. While the formation of capillary-like structures in the plasma clot was minimally affected by the addition of cRGD or endostatin alone, tube formation was largely inhibited when cRGD and endostatin were added simultaneously (Fig. 3). A similar inhibition was achieved by simultaneous addition of cRGD and mAb IIA1 (Fig. 3). These results indicate that cRGD and endostatin inhibited the formation of tubes to the same extent as mAb LM609 and endostatin.

### Tube formation in a fibrinous exudate requires interaction between fibrin and RGD-binding integrins

The plasma clot consists of various matrix proteins that contain RGD-sequences, in particular fibrin, fibronectin and vitronectin. While the RGD-sequences in vitronectin of human and mice are comparably organized, the RGD-sequences in the A $\alpha$ -chain of fibrinogen are markedly different. Therefore, we investigated whether the effects of integrin inhibitors were comparable, when hMVECs formed tubular structures in a fibrin matrix prepared from purified fibrinogen. As is shown in Fig. 4, addition of integrin inhibitors, in particular against  $\alpha 5\beta 1$ - and  $\alpha v\beta 3$ -integrins, largely reduced tube formation in a fibrin matrix. These data suggest that fibrin largely determines integrin requirement for tube formation in a plasma-derived fibrinous matrix.

## Discussion

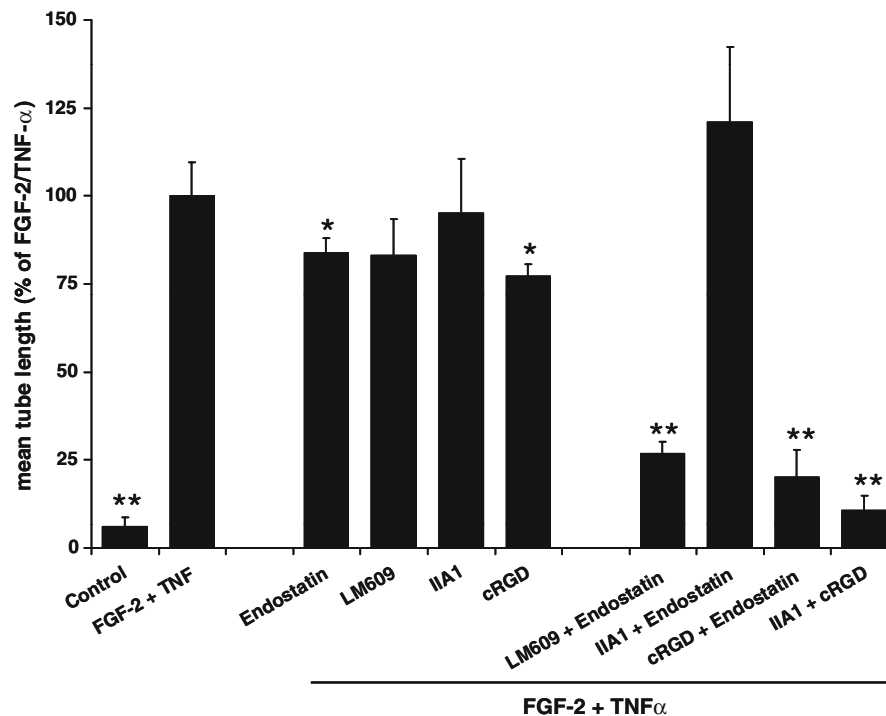
In this report we demonstrate the interplay of  $\alpha v\beta 3$ -,  $\alpha v\beta 5$ - and  $\alpha 5\beta 1$ -integrins in the formation of capillary-like structures in a 3-D in vitro plasma-derived fibrinous matrix. In the 3D-fibrinous matrix, tube formation was only largely inhibited when integrin antagonists were used together, in particular those blocking the  $\alpha v\beta 3$ - together with the  $\alpha 5\beta 1$ -integrins. Accordingly, endostatin in combination with

**Table 1** Simultaneous blocking of  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrins induces inhibition of tube formation as well as a decrease of u-PA production and FDP formation

	Tube formation (% of control)	uPA accumulation (% of control)	FDP formation (% of control)
FGF-2/TNF- $\alpha$	100	100	100
FGF-2/TNF- $\alpha$ + LM609	88.9 $\pm$ 9.4	69.1 $\pm$ 10.7	93.0 $\pm$ 9.2
FGF-2/TNF- $\alpha$ + IIA1	95.1 $\pm$ 9.5	84.2 $\pm$ 6.7	106.7 $\pm$ 22.7
FGF-2/TNF- $\alpha$ + LM609 + IIA1	22.7 $\pm$ 1.6*	56.3 $\pm$ 12.3*	58.8 $\pm$ 8.0**

HMVECs were cultured on a three-dimensional plasma matrix with FGF-2 (10 ng/ml) and TNF- $\alpha$  (10 ng/ml) to induce capillary-like tubular structures, in the absence or presence of the  $\alpha v\beta 3$ -blocking mAb LM609 (10  $\mu\text{g/ml}$ ), the  $\alpha 5$ -blocking mAb IIA1 (2  $\mu\text{g/ml}$ ) or the combination of these mAbs. After 6 days of culturing, the mean tube length of duplicate wells was quantified ( $\text{mm/cm}^2$ ) as described. The accumulation of u-PA antigen (100 = 22.6 ng/10<sup>5</sup> cells/6 days as determined by summation of three 48 h conditioned media) and the formation of fibrin degradation products (100 = 1,110 ng/10<sup>5</sup> cells in total after 6 days) were determined in the conditioned media by ELISA and expressed as percentage of the FGF-2/TNF- $\alpha$  control. The data represent mean  $\pm$  SEM of 4 independent experiments

\*  $P < 0.01$ ; \*\*  $P < 0.05$  as compared to 100% (=FGF-2/TNF- $\alpha$ -stimulated condition)



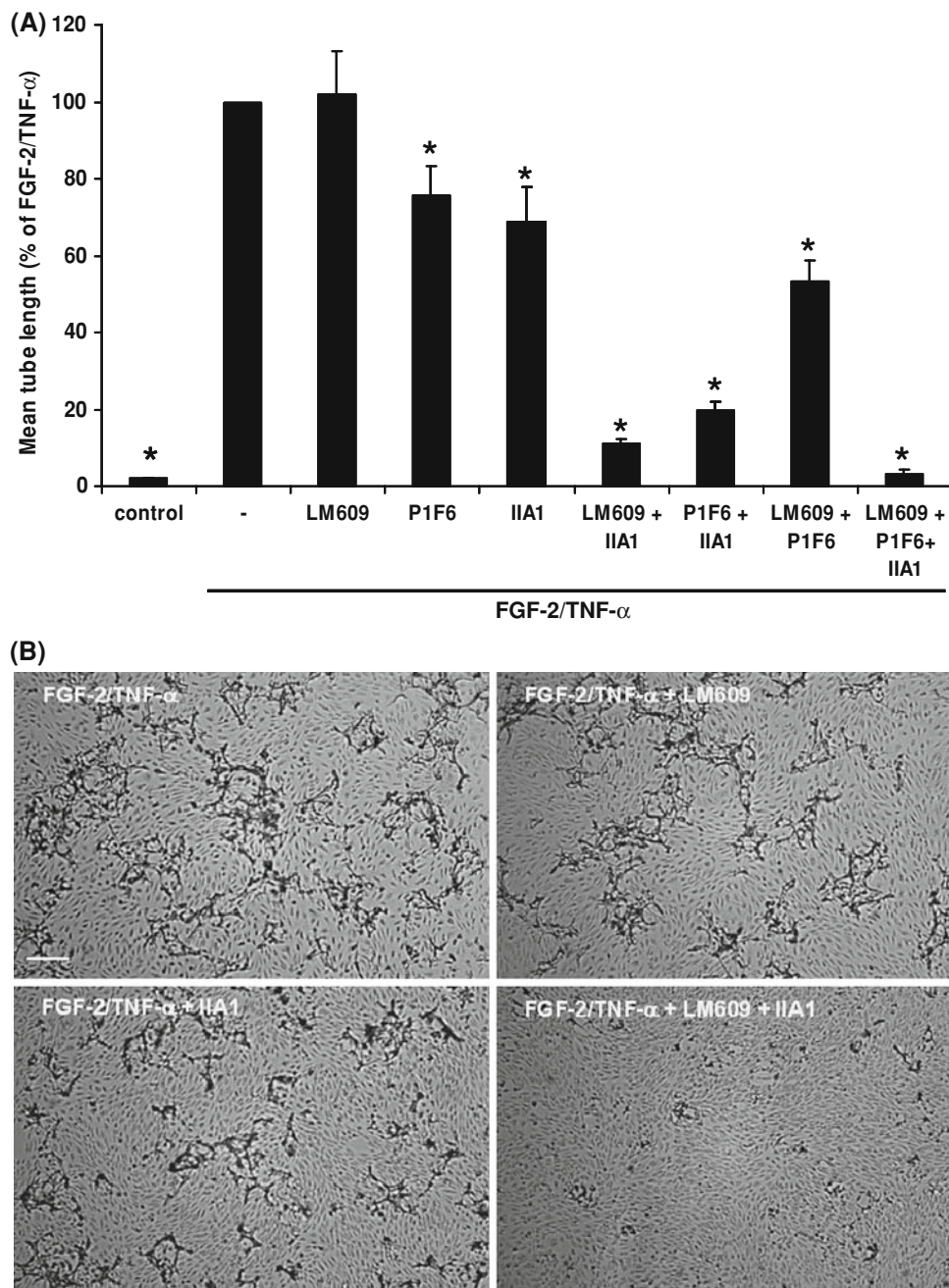
**Fig. 3** Combined effect of endostatin, LM609, and/or cRGD on capillary-like tube formation in fibrinous plasma clots. HMVECs cultured on a three-dimensional plasma clot were not stimulated (control) or stimulated with FGF-2/TNF- $\alpha$  in the presence of either endostatin (10  $\mu$ g/ml), the  $\alpha$ v $\beta$ 3-blocking mAb LM609 (10  $\mu$ g/ml), the  $\alpha$ 5-blocking mAb IIA1 (2  $\mu$ g/ml) or cRGD (50  $\mu$ g/ml), a combination of endostatin and LM609, endostatin and IIA1, cRGD

and endostatin, or IIA1 and cRGD. After 6 days of culturing, the mean tube length was quantified (mm/cm<sup>2</sup>) as described. The data represent mean %  $\pm$  SEM of FGF-2/TNF- $\alpha$  stimulated tube formation of 3–5 independent experiments. The mean tube length of FGF-2/TNF- $\alpha$  was  $98 \pm 15$  mm/cm<sup>2</sup>. \*  $P < 0.05$ , \*\*  $P < 0.01$  as compared to 100% (=FGF-2/TNF- $\alpha$  stimulated condition)

either the  $\alpha$ v $\beta$ 3-integrin inhibiting mAb LM609 or the  $\alpha$ v-competing cyclic-RGD peptide induced a dramatic decrease of tube formation, while endostatin alone had only a minimal effect. These data suggest that integrin-mediated interactions of endothelial cells with a fibrinous matrix are required for, but also support survival of, capillary tube formation by these cells.

The fibrinous exudates formed in wounds and tumor stroma are considered as supporting matrices for angiogenesis [4, 44, 45]. Our data indicate that the formation of capillary-like tubular structures in vitro proceeds similarly in a fibrin matrix prepared from purified fibrinogen and in a fibrinous exudate matrix prepared from plasma. Therefore, the focus was on the fibrin-recognizing integrins  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5 and  $\alpha$ 5 $\beta$ 1, which have been implicated in angiogenesis [15, 16, 21, 46, 47]. These fibrin-binding integrins were present on the endothelium of newly formed vessels in a recanalized mural thrombus and conform previous reports [48–50]. The RGD-integrin inhibiting agent echistatin completely inhibited the FGF-2/TNF- $\alpha$ -induced formation of capillary-like structures by hMVECs, indicating that RGD-containing integrins were essential for neovascularization [15, 16, 21, 46]. Furthermore, our data indicate that a complete inhibition of capillary-like tube formation

requires the simultaneous inhibition of these integrins, in particular the  $\alpha$ v $\beta$ 3- and  $\alpha$ 5 $\beta$ 1-integrins. These data compare well with those of integrin inhibiting antibodies on the reorganization of endothelial cells embedded in a three-dimensional fibrin matrix [51]. The mutual interaction of  $\alpha$ 5 $\beta$ 1-integrin and  $\alpha$ v-containing integrins, in particular  $\alpha$ v $\beta$ 3-integrin was underscored by using two unrelated types of inhibitors for the  $\alpha$ 5 $\beta$ 1-integrin (mAb IIA1, endostatin) and the  $\alpha$ v $\beta$ 3-integrin (mAb LM609, cRGD), which make inhibitor-specific effects less likely. Several recent reports have pointed to the induction of apoptosis in endothelial cells as a cause of angiogenesis inhibition by ligands of the  $\alpha$ v $\beta$ 3-integrin, such as mAb LM609 and tumstatin [34]. Our in vitro data did not provide direct evidence of the occurrence of such mechanism in endothelial cells that were in contact with a fibrinous matrix. This suggests that the interaction of hMVECs with the fibrin matrix may provide signals into the cells that protect them from going into apoptosis, once  $\alpha$ v $\beta$ 3-integrin is blocked. In this context, it should be noted that the adhesive potential of endothelial cells to bind fibrin(ogen) depends on transglutaminase and FXIIIa [52]. Oligomerization of the  $\alpha$ C-domains of fibrinogen by transglutaminase, stimulates their ability to bind  $\alpha$ v $\beta$ 3-,  $\alpha$ v $\beta$ 5-, and  $\alpha$ 5 $\beta$ 1-integrins on



**Fig. 4** Blocking of  $\alpha$ v $\beta$ 3- and  $\alpha$ 5 $\beta$ 1-integrins results in a combined effect on the inhibition of capillary-like tube formation in purified fibrin matrices. HMVECs cultured on a three-dimensional fibrin matrix and were not stimulated (control) or stimulated with FGF-2/TNF- $\alpha$  in the absence or presence of the  $\alpha$ v $\beta$ 3-blocking mAb LM609 (10  $\mu$ g/ml), the  $\alpha$ v $\beta$ 5-blocking mAb P1F6 (10  $\mu$ g/ml), the  $\alpha$ 5-blocking mAb IIA1 (2  $\mu$ g/ml) or the combinations of these mAbs. After 6 days of culturing, the mean tube length of duplicate wells was quantified (mm/cm<sup>2</sup>) as described (a). The data represent mean

%  $\pm$  SEM of FGF-2/TNF- $\alpha$  stimulated tube formation of 3–7 independent experiments. The mean tube length of FGF-2/TNF- $\alpha$  in the purified fibrin matrix was  $83 \pm 4$  mm/cm<sup>2</sup>. **b** After 6 days of culturing, non-phase photomicrographs were taken of hMVECs stimulated with FGF-2/TNF- $\alpha$  in the absence or presence of LM609, IIA1 or the combination of these mAbs in the concentrations described above. Bar represents 300  $\mu$ m. \* $P$  < 0.01 as compared to 100% (=FGF-2/TNF- $\alpha$  stimulated condition)

endothelial cells. The oligomerization did not only increase cell adhesion, but also promoted integrin-dependent cell signaling via focal adhesion kinase (FAK) and extracellular signal regulated kinase (ERK) [53].

Our data suggest that integrin–fibrin interactions were dominant in the plasma-derived fibrinous matrix, because a similar effect was observed with purified fibrin matrices as with plasma-derived fibrinous matrices. This directs the



attention to the RGD-sequences in fibrin, of which the RGD-sequence 572–574 in the  $A\alpha$ -chain of human fibrinogen plays a major role in the invasion of hMVECs in a fibrin matrix [54–56]. Interestingly, this RGD-sequence is absent in mouse fibrinogen (Fig. 5), while three additional RGD-sequences were present between position 272 and 286 in the murine  $A\alpha$ -chain (compare human and mouse  $A\alpha$ -chain in Fig. 5). Thiagarajan et al. [54] have demonstrated that in the bovine fibrinogen the RGD-sequence at position 272–274, which is homologous to an RGG in human fibrinogen, can mediate endothelial cell adhesion. It is likely that this substitution plays a similar role in the murine fibrinogen. However, given the importance of the flanking regions in the specificity of the RGD-sequences data from mouse and man must be compared with cautiousness. Furthermore, we can not exclude an indirect additional role for integrin  $\alpha 3\beta 1$ , which was reported to affect endothelial cell adhesion [57] and migration [58]. However, such effect will be limited, as inhibition of  $\beta 1$ -integrins alone did not significantly alter endothelial tube formation in a fibrin matrix.

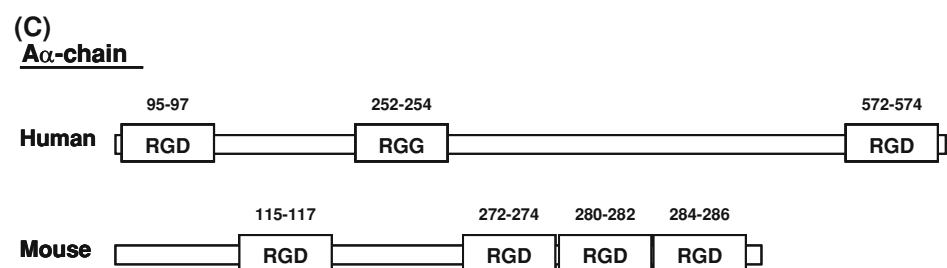
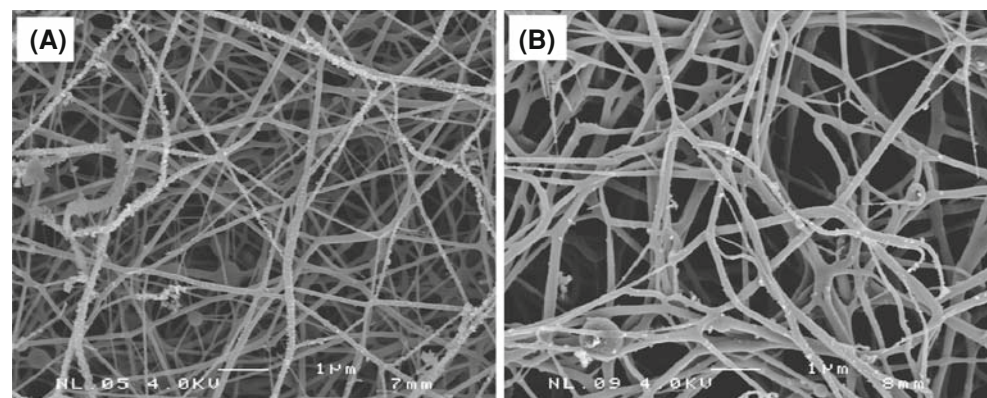
In the fibrin matrix, the inhibitory effect of endostatin is highly compatible with  $\alpha 5\beta 1$ -integrin blocking activity. Although endostatin does not contain an RGD-sequence, it can interact with RGD-binding integrins, particularly  $\alpha 5\beta 1$ -integrin [33], similar to the binding of the C-terminus of MMP-2 to RGD-dependent integrins [59].

Our in vitro data demonstrate that blocking of only one integrin is not sufficient for complete inhibition of tube formation by human MVEC in a human fibrin or plasma clot, but requires the simultaneous inhibition of  $\alpha v\beta 3$ - and

$\alpha 5\beta 1$ -integrins. Our data, as well as the recent findings of Carnevale et al. [60], support the use of a combined administration of  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrin antagonists to block angiogenesis in an experimental setting. They showed that blocking of both  $\beta 1$ - and  $\beta 3$ -integrins resulted in inhibition of tube formation in fibrin matrices using the rat aortic ring model, whereas blocking of only one of the integrins was ineffective. Using collagen matrices they found that  $\beta 1$ -integrins, but not  $\beta 3$ -integrins were required for angiogenic sprouting. These data indicate that the integrin requirements may vary for neovessel formation, dependent on the composition of the ECM. Indeed, endothelial cells overexpress  $\alpha v\beta 3$ -integrin when exposed to fibrin and during wound healing in a fibrin rich matrix, vascular cells transiently express  $\alpha v\beta 3$ -integrin [26, 61]. Moreover, Bayless et al. [62] reported that the integrin-dependent spingosine-1-phosphate regulates endothelial cell invasion, lumen formation, and branching morphogenesis in fibrin matrices, which is dependent on both  $\alpha v\beta 3$ - and  $\alpha 5\beta 1$ -integrins.

Although the involvement of particular integrin combinations, essential for angiogenesis, remains controversial, it is likely that multiple integrin attack prevents compensatory mechanisms. And our finding demonstrates the flexibility of the angiogenesis process in a temporary fibrin matrix. Moreover, it also may bear impact on the use of inhibitors of specific integrins for anti-angiogenesis treatment [63, 64]. After initial damage of the blood vessel by blocking agents of individual integrins, the formation of a fibrin exudate may support survival of endothelial cells.

**Fig. 5** Analysis of the human and mouse fibrinogen. Scanning electron microscopy of human (a) and mouse (b) plasma-derived fibrin clots. Bar represents 1  $\mu$ m. c Schematic representation of the fibrinogen  $A\alpha$ -chain in human and mouse plasma



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