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Tissue factor for influencing blood vessel formation

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(71) Applicant(s)
Merckle GmbH

(72) Inventor(s)
Peter Nawroth; Katsumi Nakagawa; Youming Zhang

(74) Agent/Attorney
DAVIES COLLISON CAVE,GPO Box 3876,SYDNEY NSW 2001

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(71) Anmelder *(für alle Bestimmungsstaaten ausser US)*: MERCKLE GMBH [DE/DE]; chem.-pharm Fabrik, Graf-Arco-Strasse 3, D-89079 Ulm (DE).

(72) Erfinder; und
(75) Erfinder/Anmelder *(nur für US)*: NAWROTH, Peter [DE/DE]; Helmholtzstrasse 26, D-69207 Sandhausen (DE). NAKAGAWA, Katsumi [JP/JP]; 4-21-201, Kagamiishicho, Okitayama, Kita-ku, Kyoto 603 (JP). ZHANG, Youming [-/DE]; Zimmer 23, Friedrich-Ebert-Anlage 51e, D-69117 Heidelberg (DE).

(74) Anwalt: HUBER, Bernard; Truderinger Strasse 246, D-81825 München (DE).

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Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist; Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(54) Title: TISSUE FACTOR FOR INFLUENCING BLOOD VESSEL FORMATION

(54) Bezeichnung: TISSUE-FAKTOR ZUR BEEINFLUSSUNG VON GEFÄSSBILDUNG

(57) Abstract

The invention relates to the use of tissue factor for influencing, in particular activating, the formation of blood vessels, above all for wound healing.

(57) Zusammenfassung

Die vorliegende Erfindung betrifft die Verwendung von Tissue-Faktor zur Beeinflussung von Gefäßbildung, insbesondere zur Aktivierung von Gefäßbildung, ganz besonders bei Wundheilung.

Abstract of the Disclosure

Tissue Factor for Influencing Blood Vessel Formation

The present invention relates to the use of tissue factor for influencing blood vessel formation, in particular for activating blood vessel formation, above all for wound healing.

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Tissue Factor for Influencing Blood Vessel Formation

The present invention relates to the use of tissue factor for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing.

The body is provided with blood by means of blood vessels. Blood vessels comprise endothelial and smooth muscle cells. In many diseases, blood vessels and the formation thereof, respectively, are impaired. This is found e.g. in impaired wound healing as in the case of diabetes mellitus, vasculitis, arterial occlusive disease, chronic venous and infected ulcer. There are also major problems in connection with wound healing in the case of innervation impairment such as paraplegia, leprosy, neuropathy, etc., and decubital gangrene of persons in need of care. Also known are weak sutures and wound healing impairment in the case of operations, particularly of the intestines and transplantations of skin or other organs, respectively. Up to the present, there are no satisfactory products or means by which it is possible to take steps in the case of blood vessel diseases, in particular impaired wound healing.

The subject matter of the present invention relates to the use of tissue factor for influencing blood vessel formation, in particular for activating blood vessel formation, above all for wound healing.

Particularly, the invention provides for the use of tissue factor or a fragment thereof for the therapeutic influence of blood vessel formation or for influencing wound healing, in tissue other than tumor or embryonic tissue, by induction of a local

expression of a tissue factor nucleic acid or by local application of a functional tissue factor protein. In addition, the invention provides for the use of tissue factor, a fragment thereof, or a nucleic acid molecule adapted to express either, in the manufacture of a composition for the therapeutic influence of blood vessel formation, or a composition for influencing wound healing, in tissue other than tumor or embryonic tissue.

The present invention is based on the applicant's finding that in wounds of animals tissue factor results in the formation of vessels (blood vessels). He found out that the vessels comprise endothelial and smooth muscle cells. He also recognized that wound healing can be achieved by means of tissue factor. Furthermore, he discovered that vessel formation can be prevented by inhibiting tissue factor.

Tissue factor is a transmembrane glycoprotein which binds the blood clotting factors VII and VIIa, respectively. An activation of the blood clotting factors X and IX, respectively, is effected by this bond, so that the blood coagulation is started via the extrinsic path and intrinsic path, respectively. Tissue factor has a molecular weight of 43 to 46 kD. Its primary structure is known as is the gene for tissue factor and its localization on the chromosome (cf. Scarpati, E.M., et al., Biochemistry 26, (1987), 5234-5238).

According to the invention tissue factor is used for activating vessel formation, particularly for wound healing. The expression "tissue factor" relates to a tissue factor of any kind and origin. It may be an animal or human tissue factor. It can be glycosylated or non-glycosylated. Also, it may be a fragment of tissue factor which is capable of forming vessels, in particular for wound healing. The tissue factor can have a wild-type sequence. Its sequence can also differ from the wild-type sequence by one or several amino acids. In addition, the tissue factor can be part of a fusion protein.

In a preferred embodiment, the tissue factor is present in the form of an expressible nucleic acid. It may be a DNA and/or RNA, a DNA, particularly a genomic or cDNA and fragments thereof, respectively, being preferred. The above statements made on the tissue factor apply here correspondingly to the nucleic acid.

The expression of the nucleic acid can be achieved as usual. It can be favorable for the nucleic acid, e.g. as a DNA, particularly cDNA, to be present in a vector which is suitable for expression in animal cells. A person skilled in the art is familiar with such expression vectors. For example, they may be virus or plasmid vectors. It is advantageous for the vectors not to integrate into the genome of cells but remain episomally within the cells. By this, a transient expression of the tissue factor is achieved, which is preferred. The nucleic acid as a DNA, particularly cDNA, can also be controlled by a constitutive or inducible promoter. An inducible promoter can be e.g. tissue-, organ- and/or tumor-specific. It can be favorable for the nucleic acid as DNA, particularly cDNA, to be controlled by the CMV promoter e.g. in the expression vector pcDNA3 (Invitrogen company) or controlled by the SV40 promoter, e.g. in the expression vector pSVK3 (Pharmacia company). Such expression plasmids referred to as pcDNA3-TF (tissue factor) and pSVK3-TF, respectively, also represent a subject matter of the present invention. It can be particularly advantageous for the nucleic acids as DNA, particularly cDNA, to be present in a Sindbis virus replicon vector. Such a vector permits an extremely high expression of the nucleic acid. An example of such a vector is the ELVS vector system from Viagene Inc. An expression plasmid referred to as ELVS-TF (tissue factor) also represents a subject matter of the present invention. For the preparation of an above vector, a person skilled in the art will use known methods. Reference is made to Maniatis, T., et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, by way of supplement.

According to the invention tissue factor is used for activating vessel formation, in particular for wound healing. The expression "vessel formation" relates to a vessel formation of any kind and at any site. For example, it relates to a vessel formation serving for replacing impaired, e.g. old, blood vessels. They can be present e.g. in the brain or heart, so that an apoplexy or infarction can

be prevented or treated. Precautions can also be taken against presbyphrenia. In addition, it relates to a vessel formation for treating arteriosclerosis, Crohn's disease and ulcerative colitis, diabetic retinopathy and deep venous thrombosis of the legs/ulcus cruris as well as the prevention of relapses. In particular, it relates to vessel formation and wound healing. The expression "wound healing" relates to wound healing of any kind and at any site. It can be normal and impaired wound healing. The latter is found in particular in the case of diseases, such as diabetes mellitus, vasculitis, arterial occlusive disease, chronic venous and/or infected ulcer as well as poorly healing gastric ulcer. Impaired wound healing is also found in the case of innervation impairment such as paraplegia, leprosy, neuropathy, etc., and decubital gangrene of persons in need of care. Impaired wound healing will also be given if weak sutures and impaired healing occur after operations, particularly of the intestines and transplantations of skin and other organs, respectively. Impaired wound healing is also found in the case of bone fractures, burns and treatments using steroids.

According to the invention tissue factor is administered in the form of a protein or an expressible nucleic acid to activate vessel formation, in particular for wound healing. It may be favorable for the tissue factor to be administered in combination with further factors supporting vessel formation, in particular for wound healing, such as vascular endothelial growth factor (VEGF). These factors can also be present in the form of proteins and/or expressible nucleic acids. The tissue factor and said factors can be administered simultaneously or successively. The kind of administration of tissue factor alone and together with said factors, respectively, can orient itself by the site of action, i.e. at the site where blood vessel formation, in particular for wound healing, shall take place. For example, it is an obvious thing to treat an area on the body surface locally and one within the interior of the body systemically. Common methods can be used for the

administration of tissue factor alone and together with said factors, respectively. For the local administration it is e.g. favorable to pack the factor or factors into liposomes or absorb them onto carriers, particularly gold particles, and apply the liposomes to the corresponding site of the body and shoot the carriers, particularly gold particles, into the tissue, respectively. Furthermore, pharmaceutical compositions are provided for the administration of tissue factor alone and together with said factors, respectively, which may contain common auxiliary substances such as carriers, solvents, etc. Such compositions also represent a subject matter of the present invention.

According to the invention tissue factor is also used for inhibiting blood vessel formation. For this purpose, the tissue factor can be present in the form of an antibody inhibiting it. The tissue factor can also be present in the form of a nucleic acid which has an antisense effect on the expression of tissue factor. In particular tumoral diseases can be treated by the inhibition of vessel formation.

By means of the present invention it is possible to influence vessel formation. In particular, vessel formation can be activated. The resulting blood vessels comprise endothelial and smooth muscle cells. Thus, the present invention is suited for the prevention and treatment of the most varying diseases. Examples thereof are indicated above. In particular, the present invention is suitable for the treatment and/or prophylaxis of impaired wound healing, above all in the case of diabetes mellitus, where it is possible to heal large open wounds located at the extremities. In addition, vessel formation can be inhibited by means of the present invention. Thus, the present invention is also suited to treat diseases, such as tumoral diseases. The present invention makes a major contribution to modern medicine.

Brief description of the drawing

Figure 1 shows the formation of vessels (blood vessels) in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tissue factor, and (c) is a control.

Figure 2 shows the formation of vessels in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tisuse factor, and (c) is a control. The blood vessels are made visible by hematoxylin/eosin staining (figure 2A). In figure 2B, the number of blood vessels is shown by way of diagram.

Figure 3 shows the presence of smooth muscle cells in newly formed vessels in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tissue factor, and (c) is a control. The muscle cells are made visible by α-actin staining (figure 3A). In figure 3B, the strength of the staining is shown by way of diagram.

The present invention is explained by the example.

Example: Preparation of a tissue factor-expressing plasmid and its use for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing

(A) The entire translated region (1.8 kb) of the mouse tissue factor gene was integrated into the BamHI site of the multiple cloning site of pcDNA3 (Invitrogen). Thus, this region was controlled by the CMV promoter. The expression plasmid pcDNA3-TF was obtained. In the same way, the coding region

(0.7 kb) of the mouse tissue factor gene was integrated in the antisense orientation into the EcoRI site of the multiple cloning site of pcDNA3. Thus, this region was also controlled by the CMV promoter. The expression plasmid pcDNA3-TF-AS was obtained.

6 mm full thickness wounds each were placed on the backs of three female NOD mice (Bomholtgaard, Denmark) at a distance of 8 to 10 mm. These wounds were treated with mixtures containing 2 µg pcDNA3-TF (a), pcDNA3-TF-AS (b) and pcDNA3 (control (c)), respectively, and 12 µg DOTAP transfection reagent (Boehringer Mannheim) each. The wounds were covered with Ohmann Opraflex.

For proving the formation of vessels (blood vessels) in the wounds, 300 µl of ink (Nigrosin, Sigma) each were injected into the caudal vein of the mice 6 days and 8 days, respectively, following the administration of the mixtures. Thereafter, the animals were killed and the skin regions with the wounds were examined under a microscope.

It showed that if a tissue factor-expressing vector (a) is administered, vessels (blood vessels) will be formed in wounds and thus wound healing will be promoted. It also turned out that an antisense tissue factor can inhibit the formation of blood vessels.

(B) As described under (A), six NOD mice were treated. After 6 days and 8 days, respectively, the animals were killed and the corresponding skin regions were examined under a microscope after having been subjected to α-actin staining (with Sm-actin antibodies from Dianova).

It showed that the blood vessels formed comprise smooth muscle cells.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement or any form of suggestion that the prior art forms part of the common general knowledge in Australia.

The claims defining the invention are as follows:

1. Use of tissue factor or a fragment thereof for the therapeutic influence of blood vessel formation in tissue other than tumor or embryonic tissue by induction of a local expression of a tissue factor nucleic acid or by local application of a functional tissue factor protein.

2. **Use according to claim 1, wherein the influence is an activation of blood vessel formation.**

3. Use of a tissue factor or a fragment thereof for influencing the wound healing in tissue other than tumor or embryonic tissue by induction of a local expression of a tissue factor nucleic acid or by local application of a functional tissue factor protein.

4. **Use according to claim 3, wherein the wound healing in the case of diabetis mellitus, vasculitis, arterial conclusive disease, chronic venous and infected ulcer, innervation impairment, decubital gangrene and weak sutures in the case of operations is concerned.**

5. **Use according to claim 1 or 2, wherein the blood vessel formation in the case of arteriosclerosis, Crohn's disease and ulcerative colitis, diabetic retinopathy and deep venous thrombosis of the legs/ulcus cruris is concerned.**

6. **Use according to claim 1 or 2, wherein the blood vessel formation for replacing impaired blood vessels is concerned.**

7. Use according to any one of claims 1 to 6, wherein the tissue factor or a fragment thereof is present as expressible nucleic acid.

8. Use according to claim 7, wherein the expression of the nucleic acid is transient.

9. Use according to claim 7 or 8, wherein the nucleic acid is a DNA.

10. Use according to any one of claims 7 to 9, wherein the nucleic acid is controlled by a constitutive or indicuble promoter.

11. Use according to any one of claims 7 to 10, wherein the nucleic acid is present in a Sindbis virus replicon vector.

12. Use according to any one of claims 7 to 10, wherein the nucleic acid is controlled by a CMV or SV40 promoter.

13. Use according to any one of claims 1 to 12, wherein the tissue factor is present in a liposome or on a carrier, particularly gold particle.

14. Use according to any one of claims 1 to 13, wherein the tissue factor is present in combination with further factors promoting the formation of blood vessels.

15. Use according to claim 14, wherein the factors are present as expressible nucleic acids or functional proteins.

16. Use according to claim 14 or 15, wherein one of the factors is VEGF.

17. Use according to any one of claims 1 to 16, wherein the tissue factor is present in a pharmaceutical composition.

18. Use of tissue factor, a fragment thereof, or a nucleic acid molecule to express either, in the manufacture of a composition for the therapeutic influence of blood vessel formation or a composition for influencing wound healing, in tissue other than tumor or embryonic tissue.

19. Use of tissue factor or a fragment thereof substantially as herein described with reference to the examples and accompanying figures.

DATED this 19th day of February, 2002

MERCKLE GMBH
By its Patent Attorneys
DAVIES COLLISON CAVE

(a)

(b)

(c)

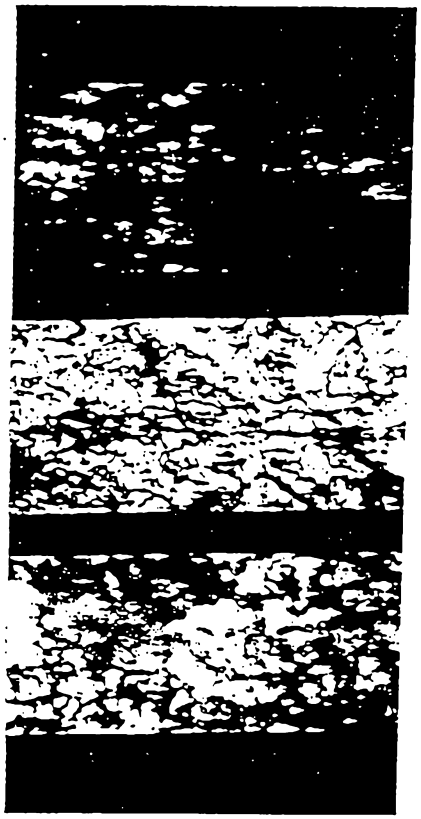
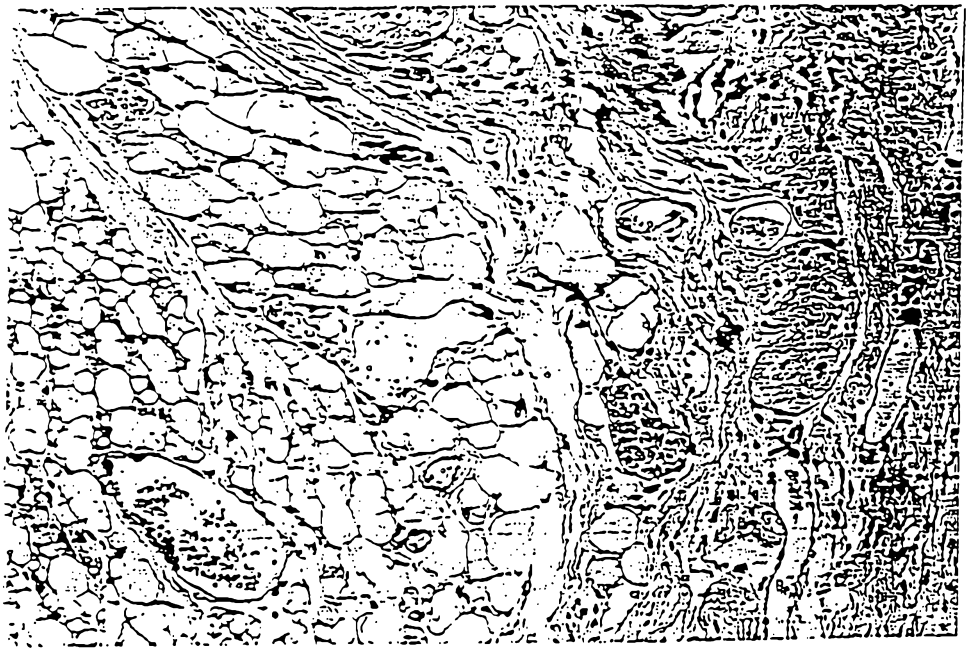


Fig. 1

(a)

(c)



(b)

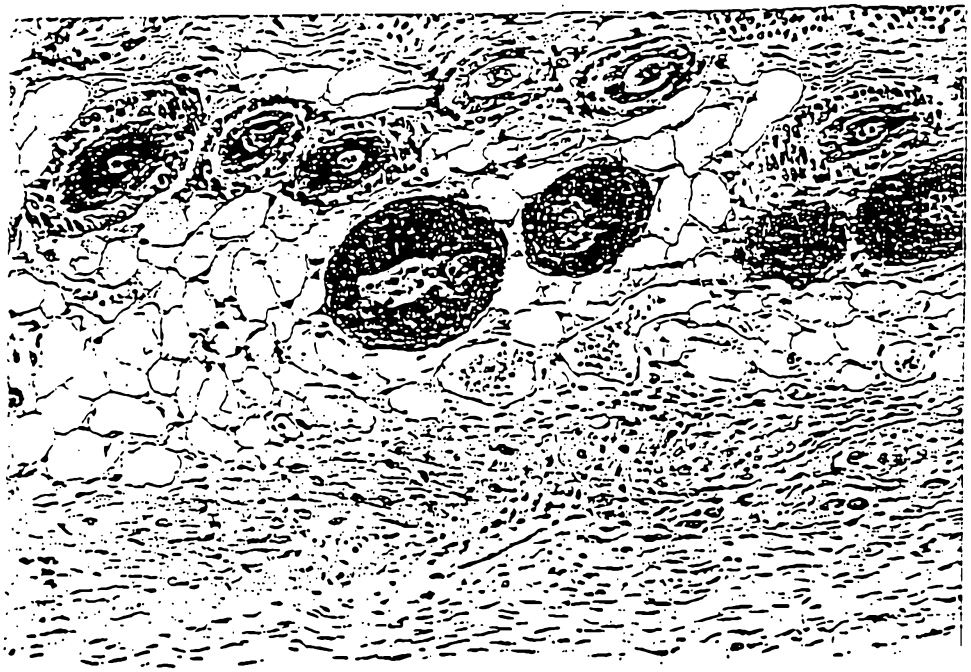


Fig. 2A

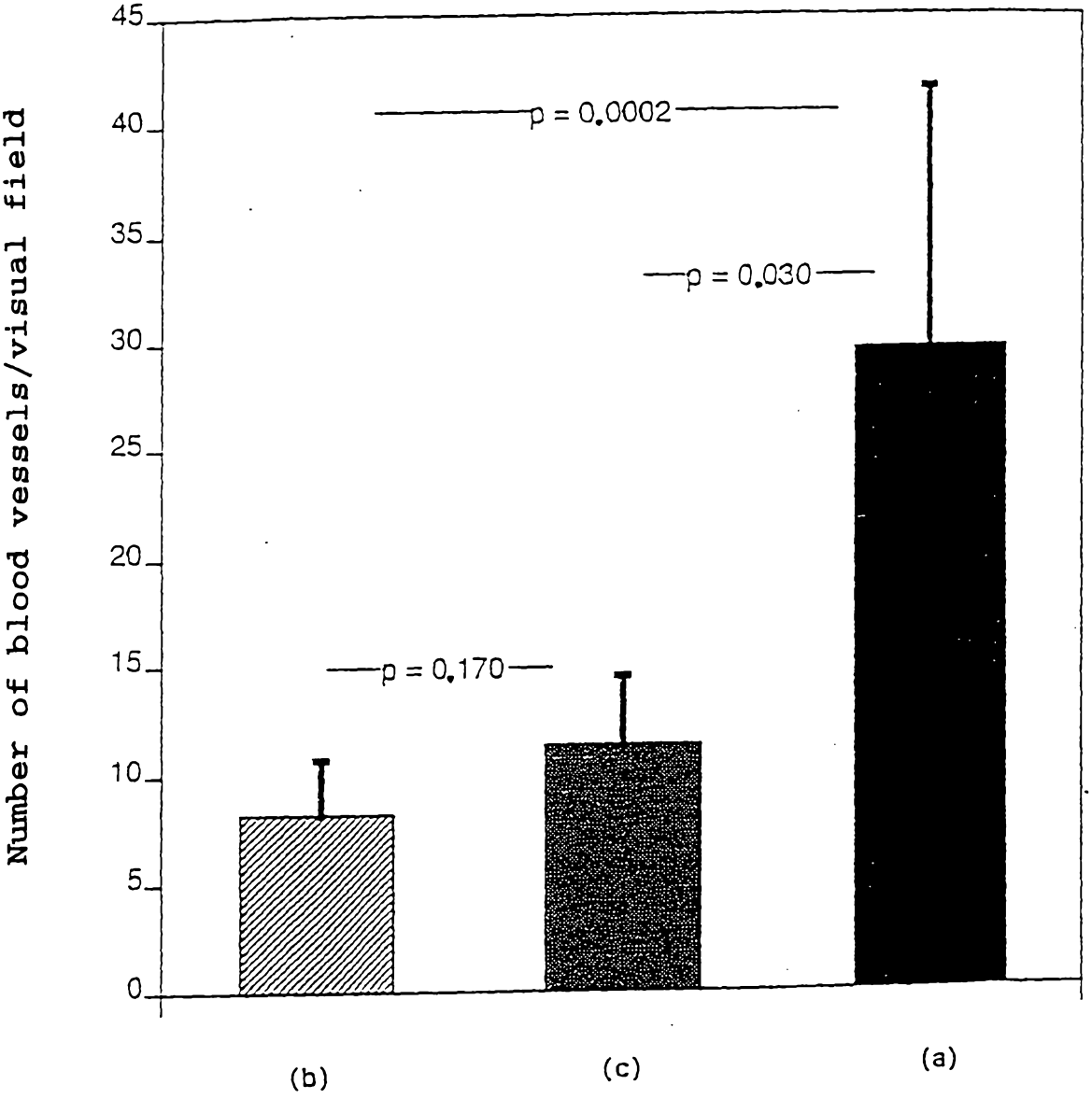
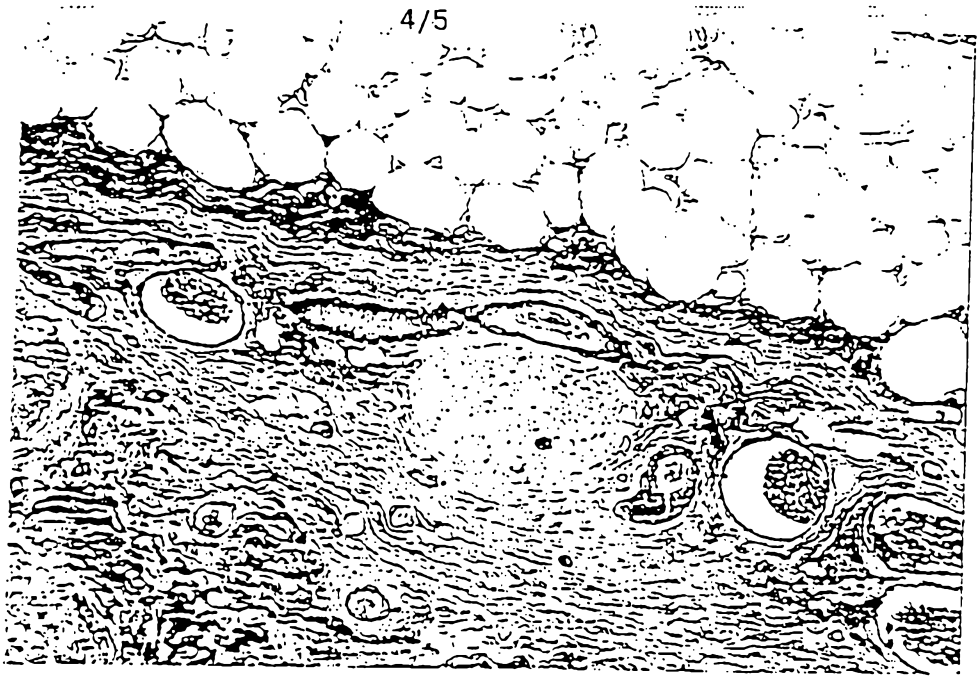
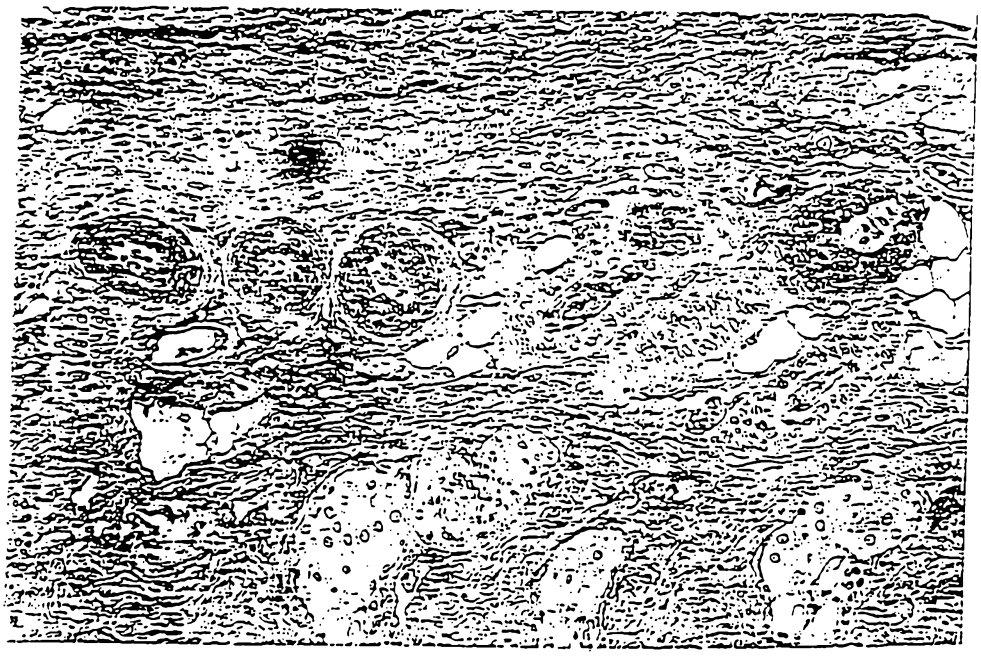

Number of blood vessels/visual field
45
40
35
30
25
20
15
10
5
0
p = 0.0002
p = 0.030
p = 0.170
(b)
(c)
(a)

Fig. 2B

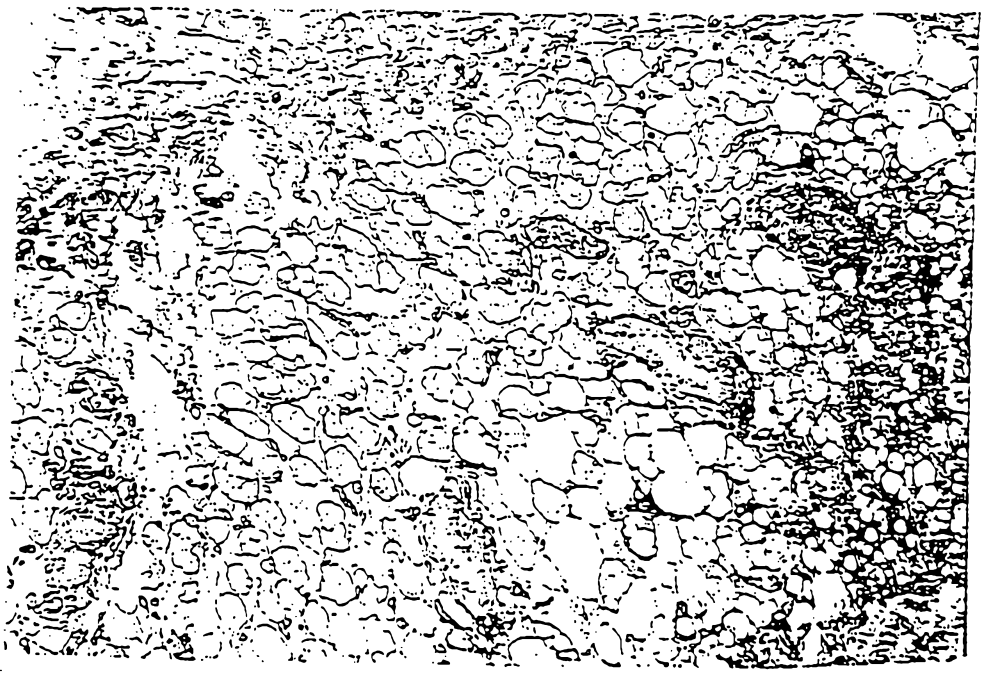
(a)

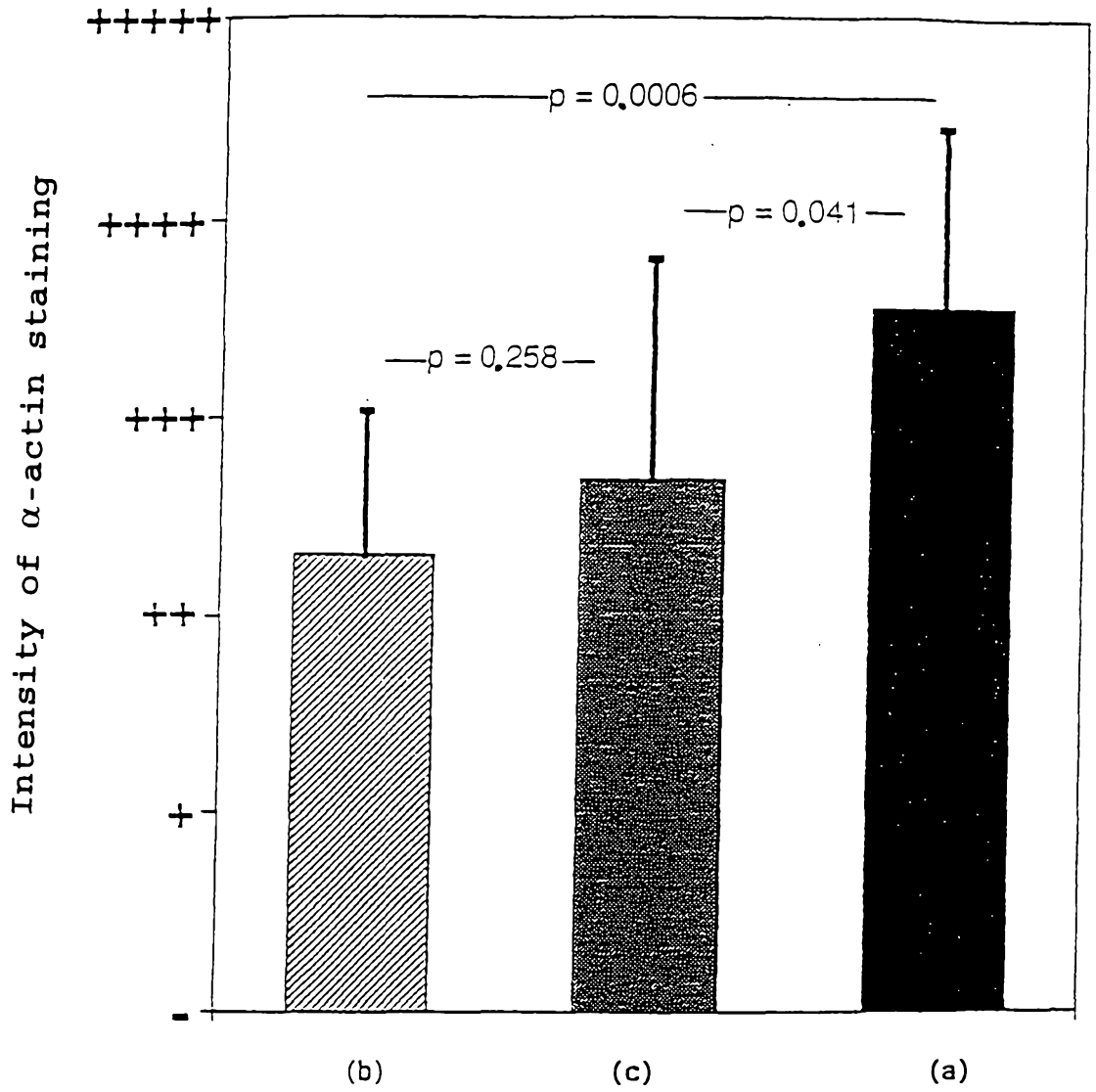


(c)



(b)





Graduation of α-actin staining:

-	=	no staining
+	=	weak staining in individual regions of the vessel walls
++	=	weak staining of the entire vessel
+++	=	partly weak, partly strong staining of the vessel wall
++++	=	strong staining of the entire vessel wall
+++++	=	very strong staining in all regions of the vessel

Fig. 3B