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Immuno-histochemical expression of α1, α2 and α3 integrin subunits during angiogenesis *in vitro*

H. Suda[1], Y. Asami[1], E. Murata[1], K. Fujita[1] and M. Akita[2]

[1]Department of Anatomy, Saitama Medical School, Saitama, Japan and

[2]Division of Morphological Science, Biomedical Research Center, Saitama Medical School, Saitama, Japan

Summary. Aortic explants were obtained from mouse fetuses and cultured in collagen gels. Immuno-fluorescence microscopy, antibodies (anti α1, α2 and α3 integrin subunits) were used. Fibroblastic cells migrated from the aortic explant after one day of cultivation. The migrating cells located in the peripheral part of the aortic explant were positive for α1 and α2 integrin subunit antibodies. Immuno-fluorescence-positive staining for the α3 integrin subunit antibody was clearly seen in the migrating cells located near the aortic explant and surrounding tube-like structures. In an immuno-electron microscope study performed by pre-embedding immuno labeling, gold particles associated with the α3 integrin subunit were found to reside on the membranes of the cells surrounding the capillary-like tubes. Two synthetic peptides, GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) and KDGEA (Lys-Asp-Gly-Glu-Ala), were added to the growth medium to study their effects on cell migration. KDGEA, a compound containing the recognition sequence for α2ß1 integrin, decreased cell migration, while GRGDSP exhibited no effect.

The migration of fibroblastic cells is an important phenomenon for tube formation. The present study suggested that the α1 and α2 integrin subunits are both involved in the cell migration, and more specifically, that the α2 integrin subunit participates in cell migration through the KDGEA sequence. The α3 integrin subunit played a role in tube formation.

Key words: Integrin, α subunit, Angiogenesis, Culture Immuno-histochemistry

Introduction

Angiogenesis, new vessel growth from pre-existing vessels, is critical to tissue development and healing. Though much is known about the molecular and cellular elements of angiogenesis, for example, the effects of growth factors and matrix molecules on proliferation and migration, it remains unclear how these elements are coordinated to produce specific microvascular beds (Hoying and Williams, 1996). In addition to requiring an angiogenic factor, *in vitro* capillary formation is also dependent on the correct extra cellular matrix (ECM) (Gamble et al., 1993). Cell surface molecules mediating adhesion to either neighboring cells or to ECM are likely to play a key role in angiogenesis. The integrins are a family of cell surface molecules that mediate the attachment of cells to the ECM and other cells. Integrins not only serve this architectural function as anchoring molecules, but also play a role as receptors for extracellular matrix proteins, a family of proteins that transduce signals from the environment into the cell and trigger various cellular behaviors such as cell spreading, migration and anchorage-dependent growth (Hynes, 1992). Integrins consist of two noncovalently associated subunits, termed α and ß. The 15 α-subunits and 8 ß-subunits so far identified are known to form 23 different αß integrin hetero-dimers in a 1:1-stochiometry, and this number may increase. These hetero-dimers differ in their ligand binding repertoires as well as in their physiological roles within cells (Eble, 1997).

Recent evidence has implicated the integrins αvß3 and αvß5 in the angiogenic process (see review of Varner, 1997). The αvß3 integrin heterodimer has been thought to play an important role in angiogenesis (Brooks et al., 1994; Ruoslahti and Engvall, 1997; Eliceiri and Cheresh, 1999). This notion has recently been called into question, however, as several groups have shown that mice lacking either the αvß3 integrin or both the αvß3 and αvß5 integrins develop normally (Bader et al., 1998; Hodivala-Dilke et al., 1999; Reynolds et al., 2002).

Our group has developed an *in vitro* model using collagen gels for studying angiogenesis (Akita et al., 1997a,b). After the fibroblastic cells migrate from the aortic explant, capillary-like tubes begin to form within the collagen gels. The migration of fibroblastic cells appeared to be an important phenomenon for this tube formation, and FGF-2 and FGF-9 were both involved in

Offprint requests to: Prof. Dr. M. Akita, Division of Morphological Science, Biomedical Research Center, Saitama Medical School, 38 Moroyama, Iruma-gun, Saitama 350-0495, Japan. Fax: +81-49-295-5998. e-mail: makita@saitama-med.ac.jp

this migration (Akita et al., 2000; Nagatoro et al., 2003). Klein et al. (1993) reported that FGF-2 increased the expression of α2ß1, α3ß1, α5ß1, α6ß1, α6ß4 and αvß5 integrins in microvascular endothelial cells. Pickering et al. (1997) found that FGF-2 promoted vascular smooth muscle cell migration, and that this promigratory effect was mediated by the upregulation of α2ß1 integrin. Langholz et al. (1995) demonstrated that α1ß1 and α2ß1 integrins were the major receptors responsible for regulating ECM remodeling in fibroblasts. Cell-ECM interaction is an important function in many biological and pathophysiological processes, including angiogenesis.

The present study examined the immunohistochemical expressions of the α1, α2 and α3 integrin subunits on the fibroblastic cells migrated from the aortic explant, as well as the influence of synthetic peptides that contained the recognition sequence for the α integrin subunits. Our results suggested that the α1 and α2 integrin subunits were involved in the cell-migration, and that the α3 integrin subunit played some role in the tube formation. This is the first report to demonstrate the localization of the α3 integrin subunit during angiogenesis by immunoelectron microscopy.

Materials and methods

Culture

Aortic explants excised from mouse fetuses (day 16 to 18 of gestation) were cultured in the collagen gels. The explants were grown in Ham's F-12 medium (Biochrom KG, Berlin, Germany) supplemented with 20% fetal calf serum (Boehringer Mannheim, Germany), 1% nonessential amino acid, 2 mM l-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Biochrom KG, Berlin, Germany). These cultures were incubated in a CO_2 incubator (95% air/5% CO_2) at 37 ºC for 2 weeks.

Collagen gels

The preparation of collagen gels has already been described by Akita et al. (1993). Briefly, a stock solution of collagen was prepared by dissolving collagen fibers (Type I, Sigma, USA) in a sterile solution of 0.2% acetic acid supplemented with 0.002% phenol red, 2.5 mg/ml, by stirring for 48 h at 4 ºC. Collagen gel was prepared by mixing 1.7 ml of collagen stock solution with 0.4 ml of a solution of Dulbecco's modified Eagle's medium (DMEM) 10X: 0.34 N NaOH 2:1 at 4 ºC. This solution was spread evenly in 35-mm culture dishes containing aortic explants, 1 ml/dish, and left to gel for 10 min at 37 ºC.

Immuno-fluorescence microscopy

Cells grown in collagen gels were fixed with 3% paraformaldehyde in PBS for 5 min, washed with PBS, incubated with the primary antibody (anti α1, α2 and α3 integrin subunits, Chemicon, CA, USA) at 4 °C for 16 hours, and incubated with the secondary antibody for 45 min: Goat anti-Mouse IgG labeled with FITC (E-Y Laboratories, Inc., CA, USA).

Transmission electron microscopy (TEM)

Cells grown in collagen gels were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), postfixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in Epon. After contrasting the thin sections with uranyl acetate and lead citrate, they were examined under a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

Immuno-electron microscopy

After fixation with 3% paraformaldehyde in PBS as described above, pre-embedded immunolabeling against integrin was performed as follows (Shakibaei et al., 1995). The cells grown in the collagen gels were incubated with the primary antibody (anti α1, α2 and α3 integrin subunits, Chemicon, CA, USA) at 4 ºC for 5 min, rinsed in PBS/BSA at 4 °C for 2x5 min, incubated with 5 nm gold labeled goat anti-rabbit IgG (H+L) (Amersham Biosciences, Japan) in growth medium at 4 ºC for 5 min, and rinsed in PBS/BSA at 4 ºC for 2x5 min. After fixing the samples in 1% glutaraldehyde at room temperature for 10 min, they were postfixed in 1% osmium tetroxide at room temperature for 30 min, rinsed in PBS, dehydrated in ethanol, and embedded in Epon. The thin sections were cut and stained with uranyl acetate and lead citrate, and the examination was performed using a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

Synthetic peptides (Yamamoto et al., 1993)

The synthetic peptide GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) that contains the cell binding sequence of fibronectin and the control peptide GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) were produced using an automated synthesizer (PSSM-8, Shimadzu, Kyoto, Japan), then further purified by reverse-phase HPLC (CLASS-10AD, Shimadzu, Kyoto, Japan). The peptide KDGEA (Lys-Asp-Gly-Glu-Ala) containing the recognition sequence for α2ßl integrin in type I collagen and the control peptide KDGAA (Lys-Asp-Gly-Ala-Ala) were produced as described above.

Effects of synthetic peptides and image analysis

To study the effect of synthetic peptides on cell migration during angiogenesis, synthetic peptides containing 2 mM Mg^{2+} were added to the growth medium at concentrations of 100 ng/ml (Yamamoto et al., 1993). The culture medium was replaced every other day during the culture period. A culture without the

addition of either of the control synthetic peptides was used as the control.

After 4 days, the cultured materials were fixed with 10% formaldehyde solution for 60 minutes, then stained by Giemsa solution for observation of the growth pattern. The cells migrated from the aortic explant were micrographed. The contact prints of the micrographs were scanned at 72 dpi (8 bits, gray scale) on a scanner (Cannon IX 4015). The distances of the cells migrated from the blood vessel stumps were measured with an image analysis application (NIH Image; version 1.52) according to our previous methods described by Fujita et al. (2002).

Results

General morphological findings

After one day in culture, spindle-shaped fibroblastic cells grew in the collagen gels from the aortic explant. After 5 days in culture, numerous fibroblastic cells were observed surrounding the aortic explant. No tubular structure was visible at this time (Fig. 1a). After 7 to 10 days in culture, capillary-like tubes were formed in the collagen gels (Fig. 1b).

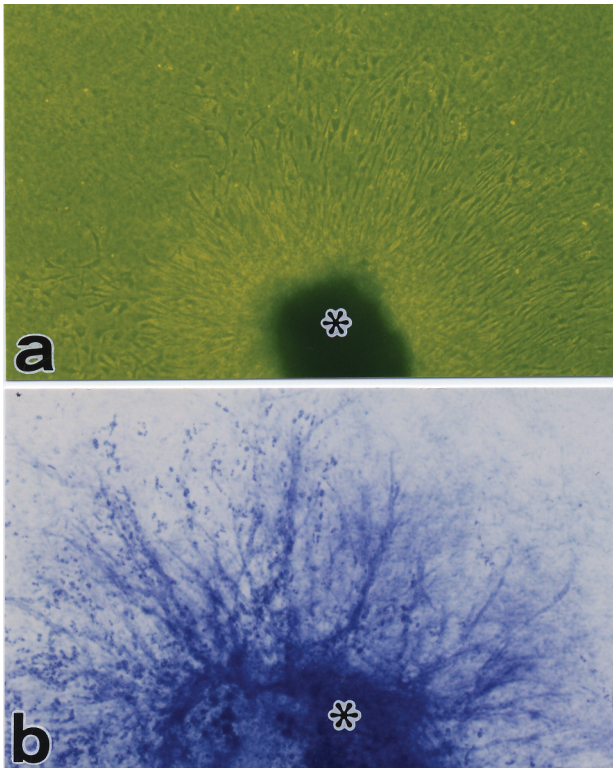

Fig. 1. a. Phase-contrast micrograph of the migrating cells into the collagen gels. After 2 days in culture, cell migration occurred from the end of the aortic explant (asterisk). Capillary-like tubes are not formed at this time. x 40. **b.** After 7 days in culture, many capillary-like tubes are seen surrounding the aortic explant (asterisk). Giemsa stain, x 40

α1 integrin subunit

Immuno-fluorescence-positive staining for the α1 integrin subunit antibody was seen in the spindle-shaped migrating cells located in outermost periphery around the aortic explant, although the inner cells were almost completely negative (Fig. 2).

α2 integrin subunit

Immuno-fluorescence-positive staining for the anti α2 integrin subunit antibody was clearly seen in the round cells located in the outermost periphery around the aortic explant (Fig. 3).

α3 integrin subunit

Immuno-fluorescence-positive staining for the anti α3 integrin subunit antibody was clearly seen in the migrating cells located near the aortic explant. A cord-like strand of cells was also positively stained (Fig. 4). Gold particles appeared on the membranes of the

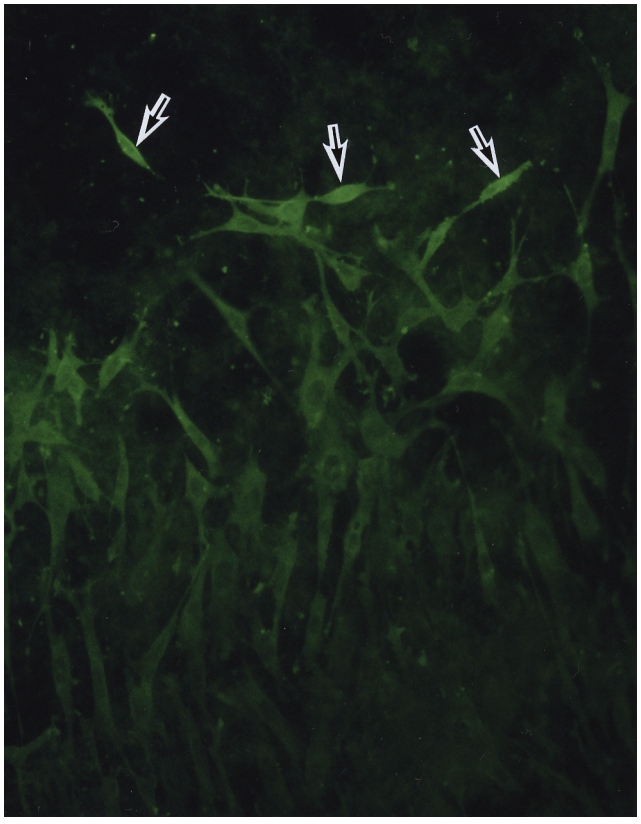

Fig. 2. Immuno-fluorescence microscopy; α1 integrin subunit. After 7 days in culture, peripheral outgrowth cells from the aortic explant within collagen gels. Immuno-fluorescent staining with anti α1 integrin antibody. The cells located in the outermost periphery were positive for the antibody (arrows). The inner cells near the aortic explant were almost completely negative. x 100

migrating cells, particularly the cells surrounding the tube-like structure with lumen. No gold particles were seen on the cell membranes of the tube-like structure with lumen (Fig. 5a-c).

Effects of synthetic peptides and image analysis

The rate of cell migration was calculated as the linear distance from the aortic explant to the tip of the migrating cells. After 4 days in culture, the synthetic peptide KDGEA decreased the rate of cell migration (Fig. 6). The influence of synthetic peptide GRGDSP could not be found. These results are summarized in Fig. 7. When the culture period was increased, no clear differences were observed in the rates of cell migration among KDGEA, GRGDSP and control. Neither KDGEA nor GRGDSP exhibited any effect in inhibiting the tube formation.

Discussion

The present study suggests that the α1 and α2 integrin subunits relate to the cell-migration and that the α3 integrin subunit plays some role in the tube formation. These three integrin heterodimers have been reported to mediate cell-type I collagen interactions. The α1 and α2 integrin subunits are known to act as major collagen receptors in most cell types, whereas the α3 integrin subunit plays a less established role as a collagen receptor (Broberg and Heino, 1996). Both α1ß1 and α2ß1 bind to native collagen, and in some cell types they can also bind to laminin, although with lower affinity (see review of Eble, 1997). The binding sites for α1 and α2 integrin subunits in type I and type IV collagen are found in the triple helical area (Gullberg et al., 1992. Eble et al., 1993). Denatured collagen can be recognized by cells via RGD-binding integrins such as α3 and αv, whereas collagen binding by α1 and α2 integrin subunits require a native conformation (Gullberg et al., 1992). The peptide KDGEA (Lys-Asp-Gly-Glu-Ala) contains the recognition sequence for α2ß1 integrin in type I collagen. Heino (1996) reported that integrin α2ß1 is required for cell migration on and invasion through collagenous matrix. In this study, the peptide KDGEA decreased the migration of fibroblastic cells from the aortic explant, suggesting that the α2 integrin subunit is involved in the cell migration through the collagenous matrix. After ligand binding, both α1ß1

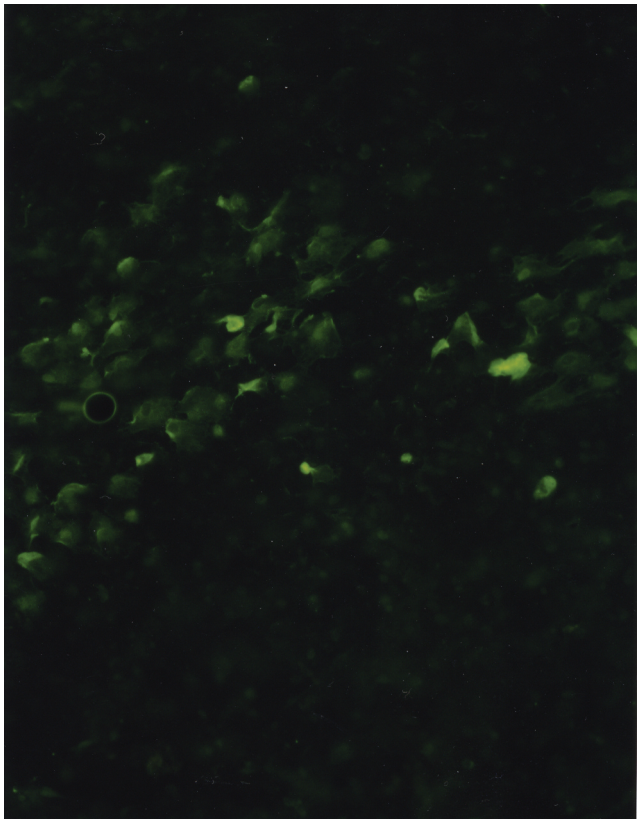

Fig. 3. Immuno-fluorescence microscopy; α2 integrin subunit. After 7 days in culture, the migrating cells located in the periphery around the aortic explant were positive for anti α2 integrin subunit antibody. x 100

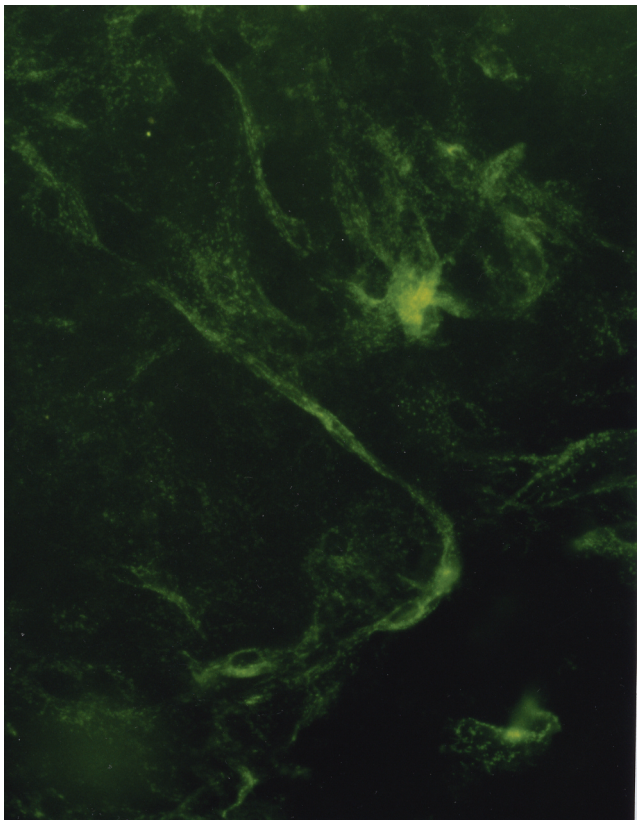

Fig. 4. Immuno-fluorescence microscopy; α3 integrin subunit. After 7 days in culture, immuno-fluorescence positive staining for anti α3 integrin subunit antibody was clearly seen in the migrating cells located near the aortic explant, and the cord like cell-strand was also positively stained. x 100

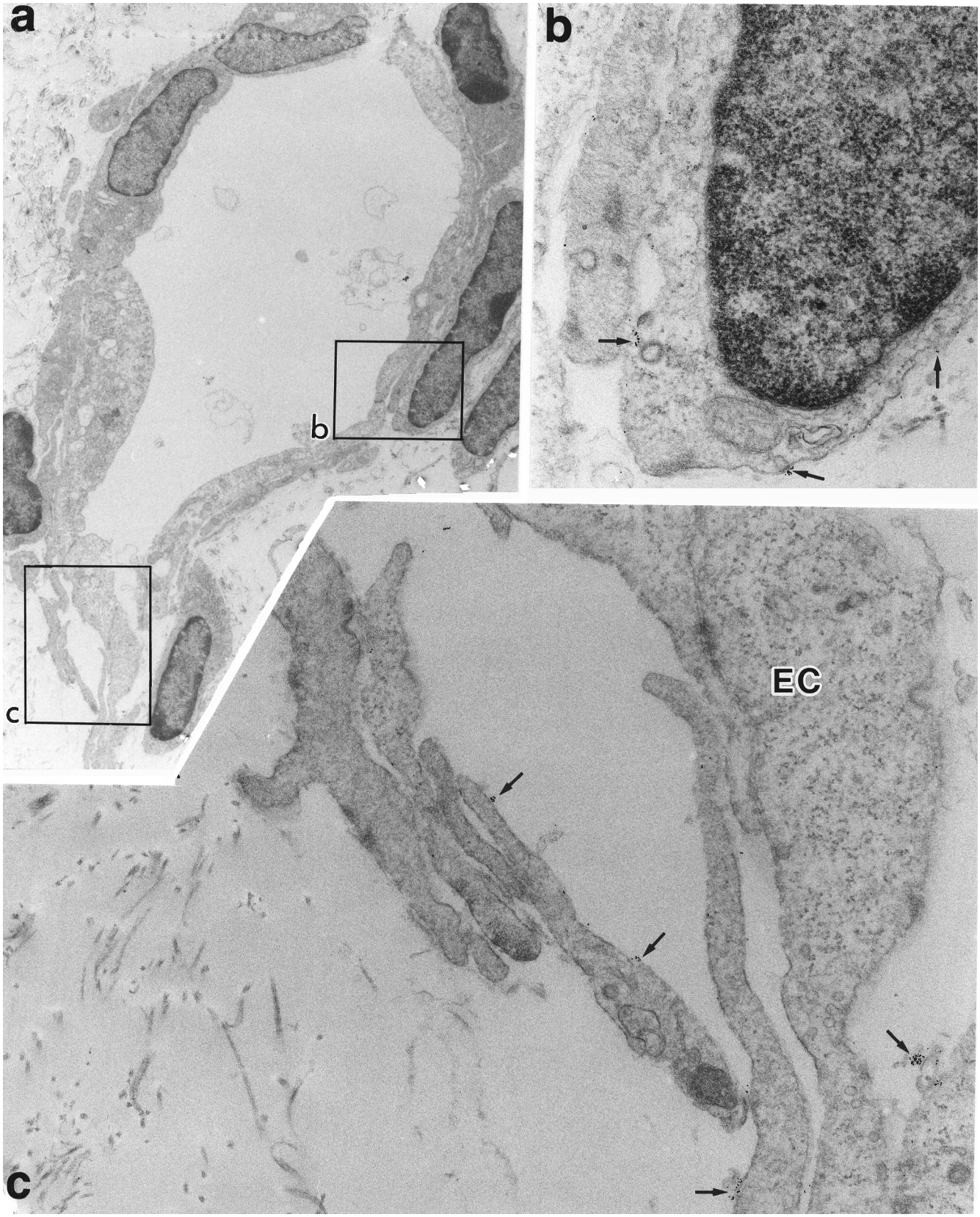

Fig. 5. Immuno-electron microscopy; α3 integrin subunit. **a.** After 7 days in culture. Some contact between endothelial cells and tube-like structures is seen. x 5,000. **b.** Higher magnification of the boxed area shown in Fig. 5-a. Gold particles (arrows) are seen in the cells surrounding the tube-like structure and foot process. x 37,500. **c.** Higher magnification of the boxed area shown by the line in Fig. 5-a. Gold particles (arrows) are seen in the cells surrounding the tube-like structure, but endothelial cells (EC) are almost completely negative. x 37,500

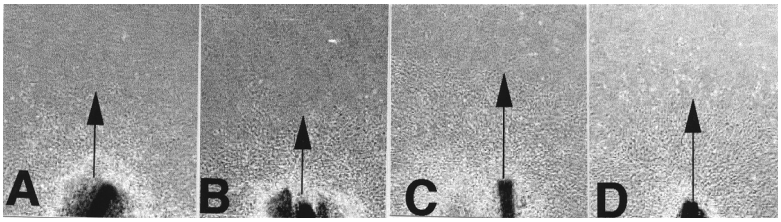

Fig. 6. Effects of the synthetic peptides KDGEA and GRGDSP. A=control (KDGAA), B=KDGEA, C=control (GRGESP), D=GRGDSP. Arrows indicate the linear distance from the blood vessel stump. x 4

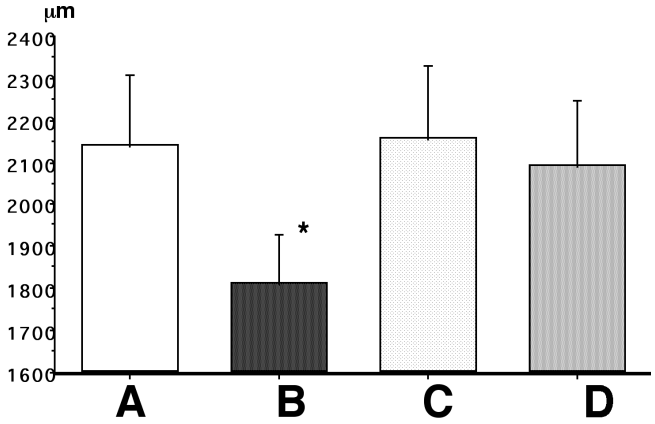

Fig. 7. Linear distance from the blood vessel stump. Data are expressed as cell-migrating distance as assessed by digital image analysis. Data represent the mean ± SD (N=10). A=control (KDGAA), B=KDGEA, C=control (GRGESP), D=GRGDSP. There was a significant difference (*$p < 0.01$) between A, control (KDGAA) and B, KDGEA treatment.

and α2ß1 trigger cellular diverse responses such as mechanical contraction of collagen gels (Schiro et al., 1991), gene activation of collagen-degrading matrix metalloproteinase-1 (MMP-1) and decreased expression of α1 (I)-chain of type I-collagen (Langholz et al., 1995; Riikonen et al., 1995). Riikonen et al. (1995) also found that α2ß1 integrin may mediate the cellular responses to extra-cellular three-dimensional collagen, including the induction of MMP-1 production. Although the native collagen molecules contain the αvß3 integrin ligands (RGD [Arg-Gly-Asp] sequences), these ligands remain masked within the triple helix and hardly bind to integrin αvß3. However, when the collagen molecules are degraded by heat or proteinases, the RGD sites are exposed and can interact with αvß3 integrin (Kuzuya, 2002). Nicosia and Bonanno (1991) found that the addition of a high concentration of GRGDS (300 mg/ml) to the culture medium brought about a marked inhibition of angiogenesis in collagen gel culture, whereas GRGES, a control peptide lacking the RGD sequence, failed to exert such an inhibitory effect. The considerably lower concentration of GRGDS (100 ng/ml) used in our study did not inhibit angiogenesis. As the RGD failed to decrease the cell migration, the α2 integrin subunit evidently played a more important role in the cell migration than the α3 and αv integrin subunits. Although the αvß1 unit has also been described as a receptor for collagen and laminin (Elices et al., 1991), it binds more readily to laminin-5 than to collagen (Carter et al., 1991; Delwel et al., 1994), and it fails to bind to a collagen-matrix (Gullberg et al., 1992). In the immuno-fluorescence microscope images from this study, the α3 integrin subunit was expressed in the cord-like cell strands. Moreover, immuno-electron microscopy revealed the expression of the α3 integrin subunit on the cells surrounding the tube-like structure with lumen. The close proximity of these cells to the basal lamina suggests that the α3 integrin subunit is closely related to tube formation. The αvß3 integrin heterodimer is believed to play an important role in angiogenesis (see the review of Varner, 1997). As previously noted, however, findings on mice lacking either the αvß3 integrin alone or both the αvß3 and αvß5 integrins have recently called these results into question (Bader et al., 1998; Hodivala-Dilke et al., 1999; Reynolds et al., 2002). Moreover, mice lacking both the αvß3 and αvß5 integrins develop more extensive tumors with richer vascular supplies than their litter mate controls (Reynolds et al., 2002). Several findings have proven that αvß3 integrin is not absolutely essential to all vasculogenesis or angiogenesis: mice and humans deficient in ß3 integrin exhibited platelet defects but nonetheless appeared to develop normal vascular beds; ß3 null mice showed normal retinal angiogenesis (Hodivala-Dilke et al., 1999); and αv knockout mice exhibiting abnormalities in brain and gut vasculogenesis developed normal vascular trees in other tissues (Bader et al., 1998). Whereas αvß3 may play a role in angiogenesis, other mechanisms must be involved or able to compensate for the lack of αvß3 (Kuzuya, 2002). Gonzalez et al. (2002) recently demonstrated that the G domain of laminin α4 chain was a specific, high-affinity ligand for the αvß3 and α3ß1 integrin heterodimers, and these ligands functioned cooperatively with α6ß1 to mediate endothelial cell-α4 laminin interaction and hence blood vessel development. Our study also suggested that the α3 integrin subunit was very important for angiogenesis. However, the α3 integrin subunit was not demonstrated on the endothelial cells with lumen, but rather on the cells surrounding the tube-like structure. In earlier studies, α3ß1 was ascribed a role in cell-cell interaction mediated by heterophilic α2ß1-α3ß1 interaction (Symington et al., 1993) and by homophilic α3ß1-α3ß1 interaction (Sriramarao et al.,

1993). Thus, further investigations will be needed to clarify the interaction of endothelial cells and surrounding pericyte cells.

In conclusion, this *in vitro* study demonstrated the expression of α1, α2 and α3 integrin subunits during angiogenesis using immuno-histochemical methods. The migration of fibroblastic cells was an important phenomenon for the tube formation. Our findings suggested that the α1 and α2 integrin subunits are both involved in the cell migration, and more specifically, that the α2 integrin subunit participates in cell migration through the KDGEA sequence. Moreover, the α3 integrin subunit played a role in the tube formation. As pointed out by Eliceiri and Cheresh (2001), analysis of the signalling pathways downstream of integrins are shedding light on the molecular basis of known anti-angiogenic strategies, as well as the design of novel therapies.

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