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ORIGINAL ARTICLE

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Synchronous appearance of fibronectin, integrin α5β1, vinculin and actin in epithelial cells and fibroblasts during rat tracheal wound healing

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Abstract The distribution of integrin $\alpha5\beta1$ ($\alpha5\beta1$) and associated components during wound healing was investigated in the rat trachea following mechanical injury. Under anesthesia, the ventral surface of the trachea was scratched, and tissue specimens were obtained from 6 h to 3 weeks after injury and studied using light and electron microscopy and immunohistochemistry. $\alpha5\beta1$, vinculin and actin in regenerating epithelial cells and extracellular fibronectin appear virtually simultaneously after injury (from 12 h to 7 days) as do $\alpha5\beta1$, vinculin and α-smooth muscle actin in fibroblasts and cellular fibronectin in granulation tissue (from 3 to 10 days). Immunoelectron microscopy 2 days after injury showed that $\alpha5\beta1$ and vinculin were localized on the basal and lateral surfaces of regenerating epithelial cells and fibroblast surfaces, and fibronectin was localized just under the regenerating epithelial cells, around collagen fibrils and sporadically around fibroblasts. Bromodeoxyuridine labeling showed that the appearance of these components was associated with the period of cell proliferation. The appearances of fibronectin, $\alpha5\beta1$, vinculin and actin in regenerating epithelial cells and fibroblasts during tracheal wound healing are well coordinated. During the initial cell migration phase, plasma fibronectin may stimulate cell migration before cellular fibronectin is produced in situ, and regenerating epithelial cells appear to begin to migrate into the wound before cell proliferation starts.

Key words Fibronectin · Integrin $\alpha5\beta1$ · Vinculin
Wound healing · Trachea

Introduction

Fibronectin receptors are members of the integrin family of cell-surface receptor complexes for extracellular matrix proteins [13, 14]. Integrin $\alpha5\beta1$ (henceforth referred to as $\alpha5\beta1$), which comprises $\alpha5$ and $\beta1$ subunits of integrin, is one of the fibronectin receptors that recognize the arginine – glycine – aspartic acid amino acid sequence (RGD) of the fibronectin cell-binding domain. Previous studies showed that fibronectin and its receptors played an important role in wound healing of the cornea [11, 22, 23] and skin [4]. Furthermore, several observations suggest there are transmembrane linkages between the cytoskeleton and extracellular matrix, and several proteins, such as vinculin [6, 10], talin [5] and α-actinin [19], have been suggested to be involved in the chain of attachment between bundles of actin filaments and the cytoplasmic face of the plasma membrane where integrin is expressed. It is important to evaluate the relationship between the extracellular matrix and these transmembrane linkages during wound healing in order to understand the mechanisms involved in normal tissue reconstruction.

In tracheal and respiratory wound healing after mechanical injury, it has been reported that the regeneration of epithelial cells begins with the migration of flattened cells derived from the marginal epithelium [18, 35, 36]. The secretory cells expel their secretory granules, then flatten and migrate into the wound [16], and fibronexus-like structures, corresponding to the linkage between extracellular matrices and actin filaments, appear in the epithelial-stromal junction before hemidesmosomes, basement membrane and anchoring fibrils appear [9, 21]. To our knowledge, there are no reports that discuss the relationship between cells and the extracellular matrix, including $\alpha5\beta1$, during the tracheal wound healing process. Therefore, we investigated the chronological changes in the distribution of fibronectin, cellular fibronectin (extradomain IIIA-containing fibronectin), $\alpha5\beta1$, vinculin and actin in epithelial cells and fibroblasts after mechanical injury of the trachea in rats. Morphometric studies of 5-bromodeoxyuridine (BrdU)-positive DNA-synthesizing cells also were performed, and the correlations between cell proliferation and the appearance of fibronectin, $\alpha5\beta1$, vinculin and actin were studied.

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Materials and methods

Specific-pathogen-free, 7- to 10-week-old male Wistar rats (Clea Japan, Tokyo), weighing 200–280 g were used in this study. They were kept in animal rooms at a constant temperature (about 22° C) with 12 h light and 12 h darkness and were provided with commercial rat chow (MS; Oriental Yeast, Tokyo) and water ad libitum. They were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (30 mg/kg), a small sterile probe was inserted transtracheally, and the ventral surface of the trachea was scratched by three longitudinal strokes from below the cricoid to the carina to cause mechanical injury of the submucosal layer. The rats were killed with an overdose of pentobarbital (i.p.) and their tracheas were removed at 6, 12, 18, 24 and 36 h and 2, 3, 4, 5, 6, 7, 10, 14 and 21 days after scratching. Each group comprised three rats. The three control rats only received pentobarbital i.p. One hour before killing, each rat was given an i.p. injection of 4 mg/100 g body weight BrdU (Becton Dickinson Immunocytometry System, Mountain View, Calif., USA).

For light microscopy the tracheas were cut into rings, fixed with 10% neutral formalin and embedded in paraffin. The paraffin-embedded tissue blocks were cut into 3-μm-thick sections, which were deparaffinized and stained with hematoxylin-eosin. Next, 3-μm-thick deparaffinized tissue sections were rinsed for 30 min with methyl alcohol containing 0.3% hydrogen peroxide to block the endogenous peroxidase activity. Then they were treated with appropriately diluted primary antibodies against fibronectin, cellular fibronectin, $\alpha 5\beta 1$, vinculin, actin, α-smooth muscle actin, laminin and type IV collagen overnight at 4° C (Table 1). The sections were washed with three changes of phosphate-buffered saline (PBS) for a total of 30 min, incubated with each biotin-labeled antibody for 60 min at room temperature, washed with PBS, and incubated with avidin biotin peroxidase complex (ABC) solution (Vectastain kit, Vector Laboratories Inc., Calif., USA) for 60 min. The peroxidase activity was detected by incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide, followed by final light counterstaining with Mayer's hematoxylin. For the detection of BrdU-positive nuclei, 3-μm-thick deparaffinized tissue sections were treated with 0.1% proteinase in 0.01 M PBS and with 2N HCl for 20 min at 37° C. After washing in 0.1 M borax buffer, pH 9.1, and PBS, the sections were incubated in 0.3% hydrogen peroxide-containing methanol for 30 min to block the endogenous peroxidase activity. Then they were incubat-

Table 1 Antibodies used for immunohistochemistry

Antibody	Mono poly	Dilution	Source
Fibronectin	Poly	1:2000	Dakopatts
Cellular fibronectin	Mono	1:300	Locus
Integrin $\alpha 5\beta 1$	Poly	1:1000	Chemicon International
Vinculin	Mono	1:2000	Sigma Immunochemicals
Actin	Mono	1:300	Biomedical Technologies
α Smooth muscle actin	Mono	1:300	Dakopatts
Type IV collagen	Mono	1:300	Shiseido
Laminin	Mono	1:300	E-Y Laboratories

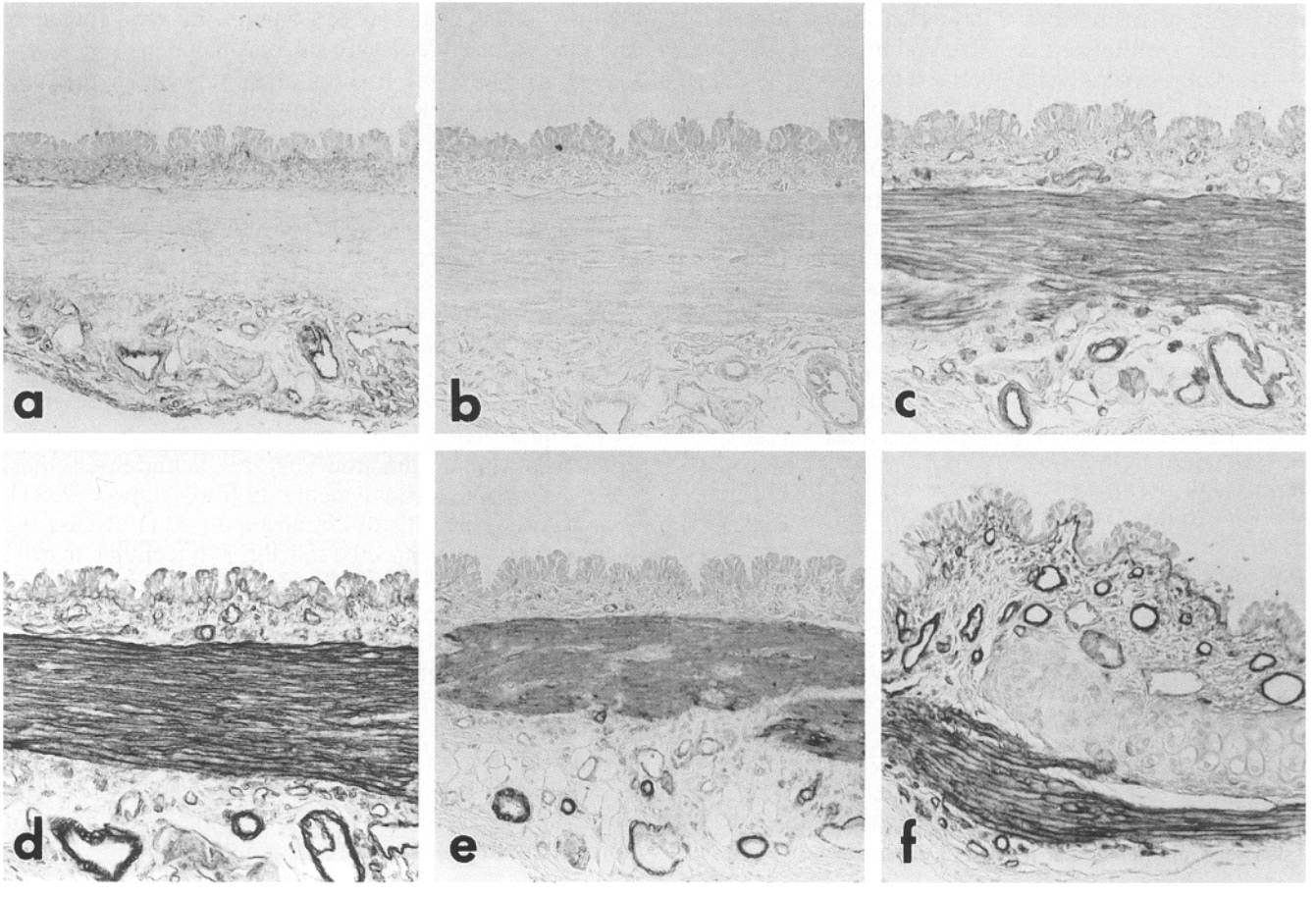
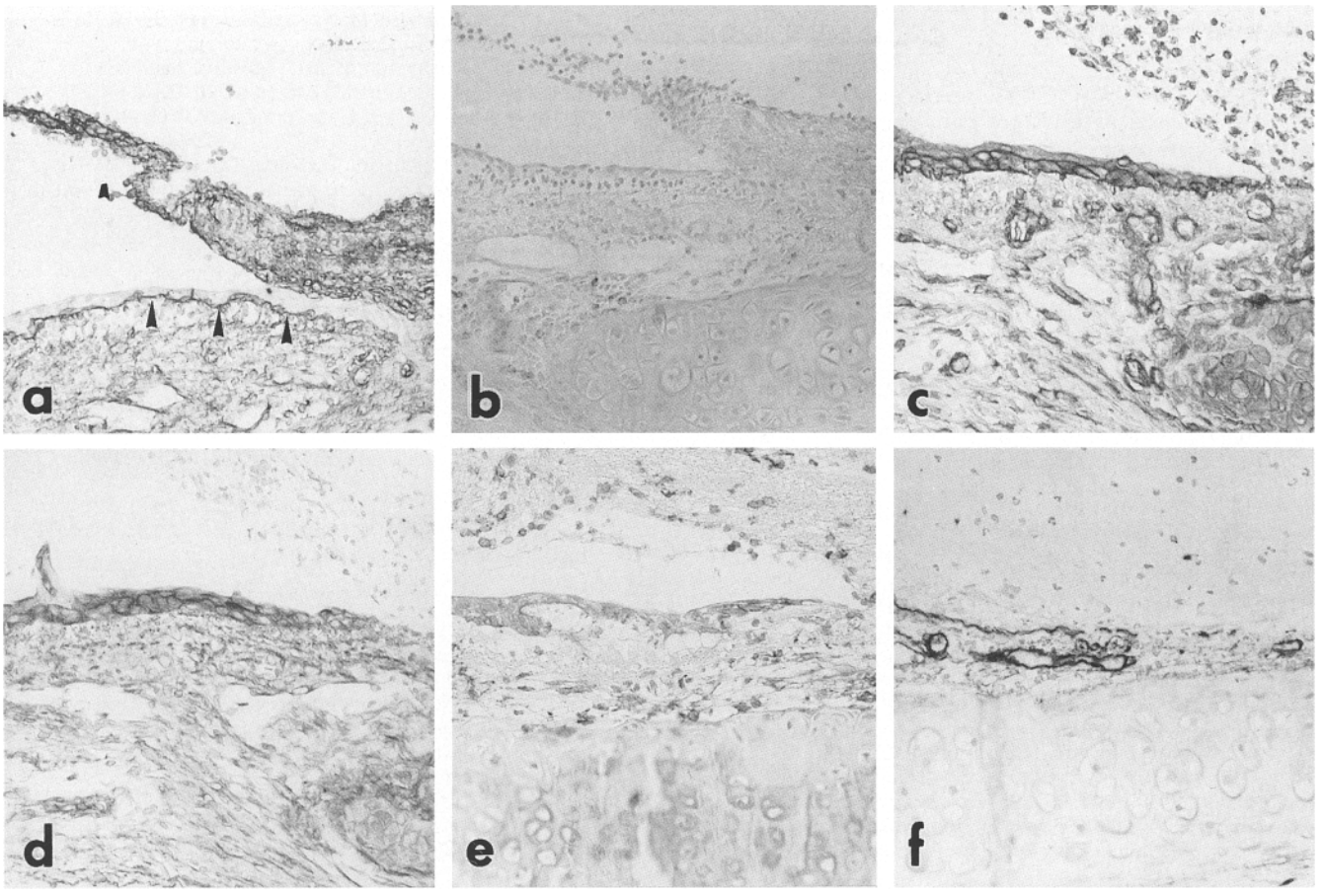


Fig. 1a–f Control rats (not injured). **a** Weak fibronectin-positive immunostaining can be seen just under the epithelium and vascular endothelium. **b** No cellular fibronectin is present. **c** The surfaces of the smooth muscle and vascular endothelial cells are $\alpha 5\beta 1$ positive. **d** Vinculin-positive staining of smooth muscle cells, vascular endothelial cells and the apical sides of ciliated cells. **e** The smooth muscle cells are actin positive. **f** The epithelial, smooth muscle and vascular endothelial basement membranes are type IV collagen positive. ×200



ed in normal horse serum, incubated overnight at 4° C with a monoclonal antibody diluted 1:100 with PBS against BrdU (Becton, Dickinson) and processed as described before [29].

In order to calculate the BrdU labelling index, the numbers of BrdU-positive and BrdU-negative nuclei in epithelial cells and submucosal fibroblasts were counted using light microscopy at a magnification of × 600. At least 1000 nuclei of each cell type per animal were counted, but if less than 1000 fibroblasts were present, the number found was multiplied by the appropriate factor and a divisor used. Any cells that could not be typed were excluded from the calculation. Statistical analysis was performed using Student's *t*-test.

For immunoelectron microscopy, tracheal tissue blocks prepared 2 days after injury were fixed in periodate-lysine-paraformaldehyde solution for 6 h at 4° C, then washed with graded concentrations of sucrose in PBS (5, 10 and 15%), treated with PBS containing 20% sucrose and 5% glycerol, embedded in OCT compound (Miles, USA), frozen rapidly and stored at –70° C until required for use. Frozen tissue sections were cut at 8–10 µm, mounted on glass slides, washed with three changes of PBS containing 10% sucrose for a total of 20 min at 4° C and incubated with 10% normal serum for 10 min. The samples were washed with PBS containing 10% sucrose, treated with the required primary antibody, diluted appropriately overnight at 4° C, washed with PBS containing 10% sucrose for 60 min at 4° C and incubated with the required horseradish peroxidase-labeled second antibody overnight at 4° C. Then the sections were washed with PBS containing 10% sucrose, incubated with 1% glutaraldehyde in PBS and washed with PBS. This was followed by incubation with 0.05 M Tris buffer (pH 7.6) containing DAB for 30 min and the peroxidase activity was detected by incubation with DAB-hydrogen peroxide solution for 3–5 min. Finally, the sections were washed with PBS, fixed with 2% osmium tetroxide in PBS, dehydrated, embedded in Epok 812 (Nagase, Tokyo), and ultrathin sections not counterstained were observed under a Hitachi 7100 electron microscope.

Fig. 2a–f Two days after injury (early stage). **a** The regenerating epithelial cells are flattened and migrating into the wounds. The exudate and stroma are fibronectin positive. Note the linear reaction just under the regenerating epithelium (*arrowheads*). **b** No cellular fibronectin is present. **c** The regenerating epithelial cell and fibroblast surfaces are $\alpha 5\beta 1$ positive. **d** The regenerating epithelial cells and fibroblasts are vinculin positive. **e** Actin is positive in regenerating epithelial cells and fibroblasts. **f** Type IV collagen-positive immunostaining of the residual basement membrane is seen. ×200

Results

Immunohistochemical studies

Control rats

Microscopic examination showed that the epithelial cells comprised ciliated, secretory and basal cells. Slight fibronectin-positive immunostaining of the stroma under the epithelium and vascular endothelial cells was observed (Fig. 1a) but no cellular fibronectin was detected (Fig. 1b). $\alpha 5\beta 1$-positive staining of the surfaces of tracheal smooth muscle cells, vessels, and endothelial cells was observed (Fig. 1c). Similar vinculin-positive staining of the smooth muscle and vascular endothelial cells and faint staining of the apical sides of the ciliated cells occurred (Fig. 1d), and smooth muscle and endothelial cells were actin positive (Fig. 1e). Both laminin- and

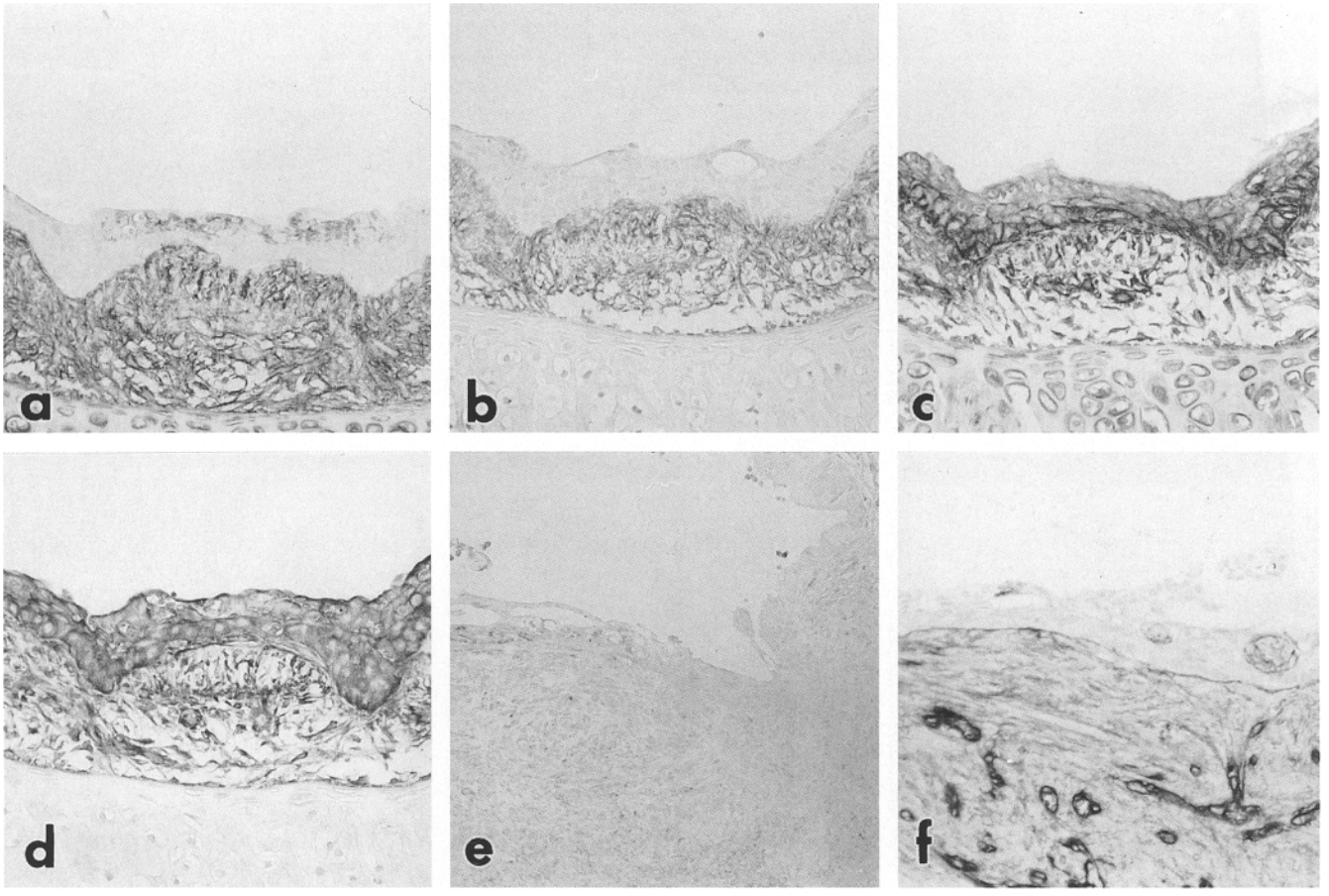


Fig. 3a–f Six days after injury (intermediate stage). **a** The wound is covered with regenerating epithelium showing squamous metaplasia, and granulation tissue has formed which shows intense fibronectin-positive staining. **b** Cellular fibronectin-positive staining of the granulation tissue can be seen. **c** $\alpha5\beta1$ is localized on epithelial basal cells and fibroblasts in the granulation tissue. **d** Vinculin-positive staining in regenerating epithelial cells and fibroblasts in granulation tissue. **e** The actin reaction in the epithelium is weaker than during the early stage. **f** Sporadic type IV collagen-positive staining just under the regenerating epithelium. ×200

type IV collagen-positive staining was observed in the basement membranes of epithelial, smooth muscle, and endothelial cells (Fig. 1f), and the smooth muscle cells were α-smooth muscle actin-positive.

Experimentally wounded rats

Early stage (6 h–2 d)

The single-layered, flattened epithelium extended to the wounded area in the early stage (6 h to 2 days) and the intratracheal exudate and stroma under the migrating epithelial cells were fibronectin positive. Linear fibronectin staining of the basal layer of the regenerating epithelial cells was observed (Fig. 2a), but no cellular fibronectin staining occurred at this stage (Fig. 2b). The basal and lateral surfaces of the migrating epithelial cells and the fibroblasts were slightly $\alpha5\beta1$ positive (Fig. 2c). The vinculin staining pattern generally resembled that of $\alpha5\beta1$ (Fig. 2d). Actin-positive immunostaining along the basal layer and occasionally the intercellular area of the migrating epithelial cells occurred (Fig. 2e), but the wounded stromal fibroblasts were α-smooth muscle actin negative at this stage. The residual basement membrane was laminin and type IV collagen positive, whereas neither was detected in the wounded area (Fig. 2f).

Intermediate stage (3–7 d)

By 3 days after injury, approximately half the wounded area was covered with regenerating epithelium, which showed squamous metaplasia, and about 5 days after injury the wounded area was completely covered with regenerating epithelium. The number of fibroblasts in the wounded area increased at this stage, and granulation tissue had formed. The granulation tissue covered with the regenerating epithelium was fibronectin positive (Fig. 3a). Cellular fibronectin-positive staining was observed in the granulation tissue, mainly around the fibroblasts (Fig. 3b). $\alpha5\beta1$ was located mainly on the surfaces of the metaplastic epithelial basal layer cells (Fig. 3c). The vinculin staining pattern was similar to that of $\alpha5\beta1$ at this stage, but all the metaplastic epithelial layers tend to be vinculin positive (Fig. 3d). Intense $\alpha5\beta1$- and vinculin-positive staining of the granulation tissue fibroblasts was observed, and, moreover, some fibroblasts in

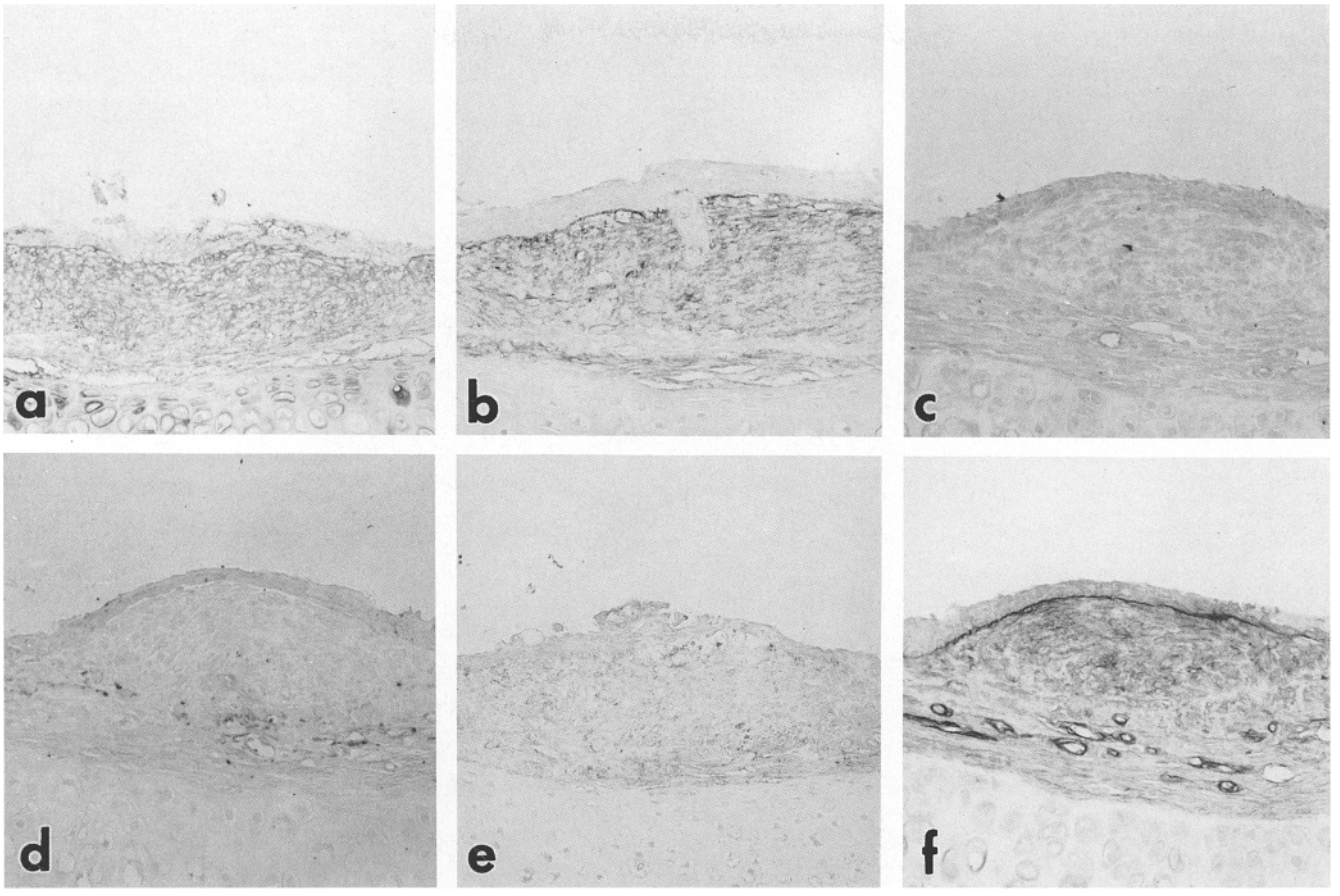


Fig. 4a–f Ten days after injury (late stage). The regenerating epithelium is differentiating into mature epithelium. The fibronectin (**a**) and cellular fibronectin (**b**) reactions in the granulation tissue are weaker than those during the previous stage, as are those for $\alpha 5\beta 1$ (**c**) and vinculin (**d**) in the epithelium and granulation tissue. **e** The epithelium is actin negative at this stage. **f** The renewed basement membrane of the regenerating epithelium shows continuous type IV collagen-positive staining. ×200

the granulation tissue at this stage were α-smooth muscle actin positive. When the wound was completely covered with regenerating epithelium, the staining of actin in epithelial cells became faint (Fig. 3e). From approximately 5–6 days after injury, linear or patchy laminin and type IV collagen staining under the metaplastic epithelium was observed (Fig. 3f).

Late stage (10–21 d)

Ten days after injury, the regenerating epithelium showed a few ciliated cells and secretory cells near the wound margin, and after 14 days it appeared similar to that in control rats. However, in tissues in which the wound healing process was prolonged, squamous metaplasia was still observed at the wound margins. In this stage, faint fibronectin-positive staining of the granulation tissue was observed (Fig. 4a) and the cellular fibronectin staining of the granulation tissue also was weaker than before (Fig. 4b). The regenerating epithelial basal layer and fibroblasts in the granulation tissue were faintly $\alpha 5\beta 1$ positive 10 days after injury (Fig. 4c), and after 14 days the appearance was similar to that in control rats. The vinculin staining pattern was similar to that of $\alpha 5\beta 1$ (Fig. 4d) and after 14 days was identical to that in the controls. Actin was negative in epithelium (Fig. 4e). Positive laminin and type IV collagen linear staining just below the regenerating epithelium, at the basement membrane and, faintly in the granulation tissue were observed (Fig. 4f). The results of the relative immunostaining intensities of $\alpha 5\beta 1$ and associated components are summarized in Fig. 8b.

Immunoelectron microscopic study

Two days after injury, immunoelectron microscopy showed that fibronectin was localized just under the regenerating epithelial cells and stroma before basement membrane renewal and occurred sporadically around the fibroblasts. The rough endoplasmic reticulum in the regenerating epithelial cells and fibroblasts was fibronectin positive at this time (Fig. 5); diffuse $\alpha 5\beta 1$ staining of the regenerating epithelial cell basal and lateral surfaces and sporadic staining of the epithelial and fibroblast cell surfaces and nuclear membranes was observed (Fig. 6). Vinculin was localized on the regenerating epithelial cell

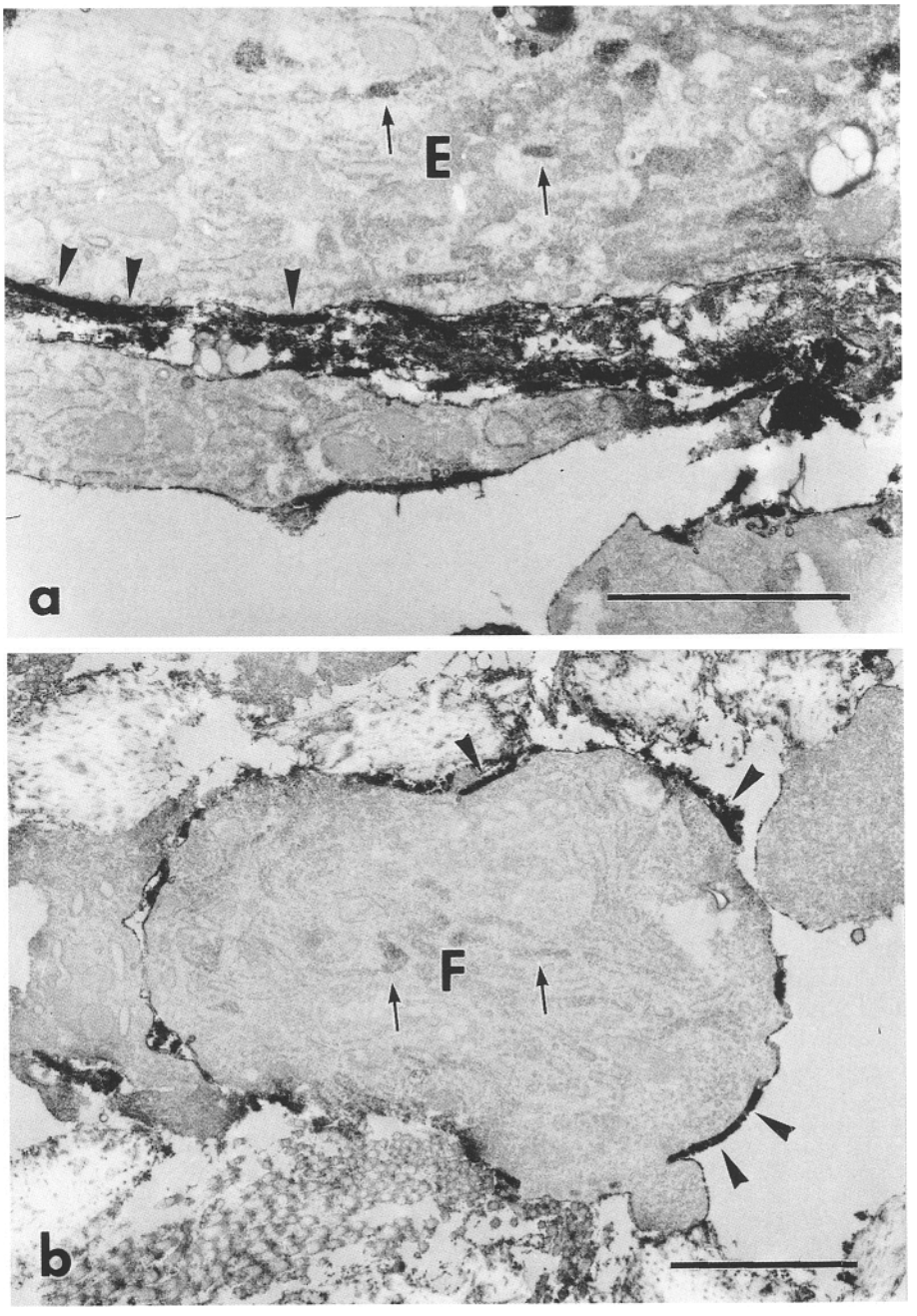


Fig. 5a, b Immunoelectron micrograph for fibronectin (early stage). Two days after injury, fibronectin is localized just under the regenerating epithelial cells (*E*, *arrowheads*) and stroma before basement membrane renewal (**a**) and occurs sporadically around the fibroblasts (*F*, *arrowheads*; **b**). The rough endoplasmic reticulum in both regenerating epithelial cells and fibroblasts is fibronectin positive (*arrows*). *Bars* 2 μm

surfaces where actin filaments had accumulated and at fibroblast peripheries (Fig. 7).

Labeling index of BrdU-positive nuclei

Figure 8a shows the BrdU labelling index of each cell type. The epithelial cell index had increased by 12 h after injury and the maximum value (approximately 40%) was observed at 18 h, after which it decreased gradually and was normal by 14 days. Statistical analysis showed that the index of regenerating epithelial cells 12 h to 5 days after injury differed significantly from that of the controls ($P<0.05$). In fibroblasts, the index increased gradually from 24 h after injury and the maximum value (approximately 15%) was observed after 2 days. It decreased slowly thereafter and was normal by 14 days after injury. Two to 6 days after injury, the fibroblast BrdU labeling index differed significantly from that of the controls ($P<0.05$), which was about 2–3% or less in both epithelial cells and fibroblasts.

Discussion

In this study, we demonstrated the synchronous appearance and distribution of fibronectin, $\alpha 5\beta 1$, vinculin and actin in epithelial cells and fibroblasts during the tracheal

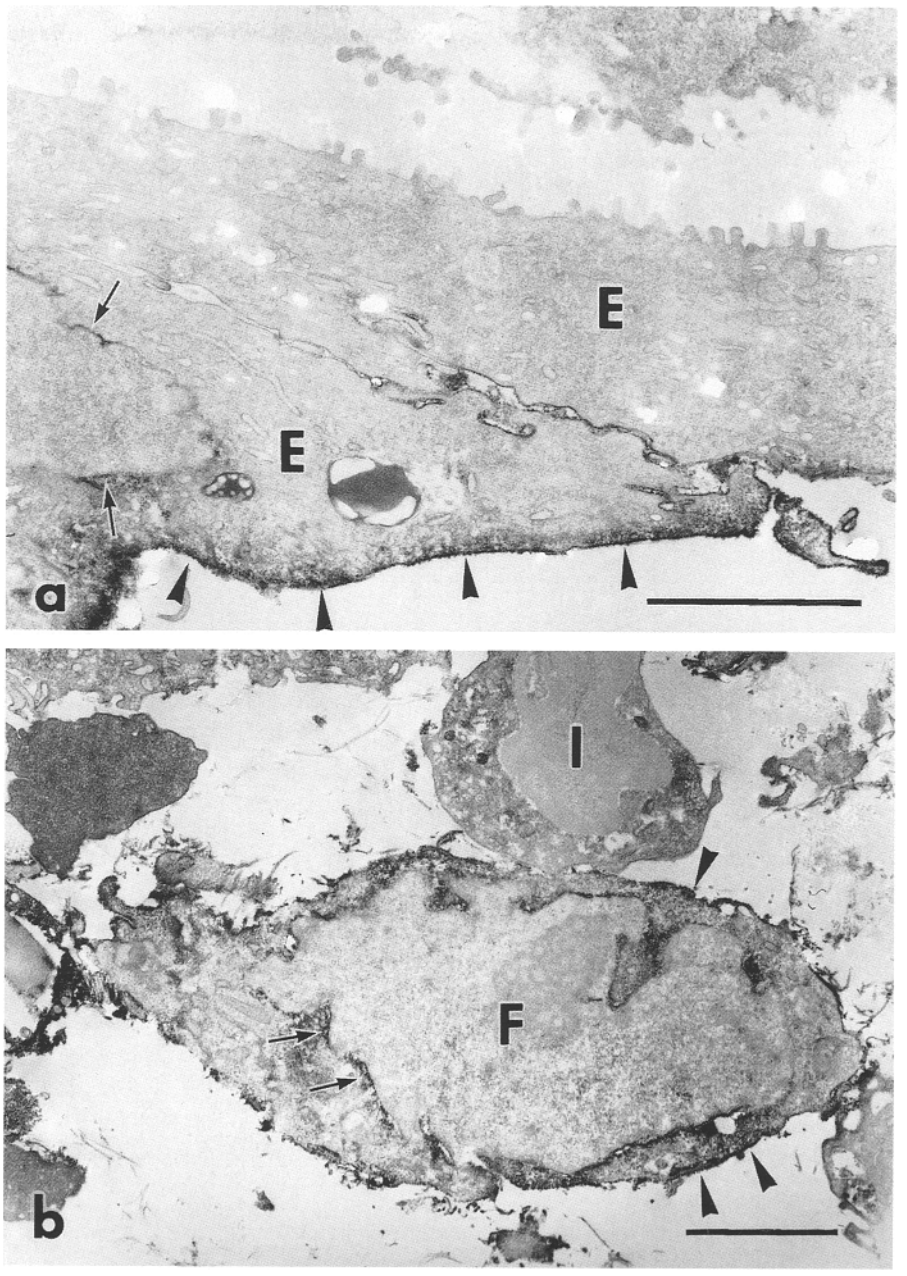


Fig. 6a, b Immunoelectron micrograph for $\alpha 5\beta 1$ (early stage). Two days after injury, $\alpha 5\beta 1$ is present on the basal and intercellular surfaces of regenerating epithelial cells (*E*, *arrowheads*; **a**) and on fibroblast cell surfaces (*F*, *arrowheads*; **b**), and can be seen on the regenerating epithelial cell and fibroblast nuclear membranes (*arrows*). The inflammatory cell (*I*) adjacent to the fibroblast is $\alpha 5\beta 1$ negative. *Bars* 2 μm

wound healing process after mechanical injury in rats. The appearance of these components was well co-ordinated spatiotemporally. Furthermore, their appearance in both types of cell was associated with the cell proliferation period, which corresponded to their BrdU labelling indices.

The distribution and appearance of fibronectin, $\alpha 5\beta 1$, vinculin and actin in the migrating epithelial cells correspond to the fibronexus-like structures which appear at the basal surface of regenerating epithelial cells in cases of lung fibrosis caused by paraquat [9] and in the mechanically injured trachea [21]. Fibronexus structures are believed to be responsible for the initial adhesion of the cells to the underlying connective tissue [9, 27, 28]. Epithelial cell migration is an important feature of wound healing, and previous studies demonstrated that components of extracellular matrix stimulate migration of epithelial cells [25, 31]. Furthermore, fibronectin promotes rabbit cultured corneal epithelial cell migration in a concentration-dependent manner [8, 23] and has a chemotactic effect on bronchial epithelial cells [26].

As the tracheal wounding in our experimental model destroyed the epithelial basement membrane, our results suggest that the initial migration of the regenerating epithelial cells into the wounded area depended on fibronectin at first, because neither type IV collagen nor laminin was observed under the migrating epithelial cells during the early stage, and the fibronectin receptor, $\alpha 5\beta 1$, appeared at the basal surfaces of regenerating epithelial cells during this stage. In a recent study, fibronectin-

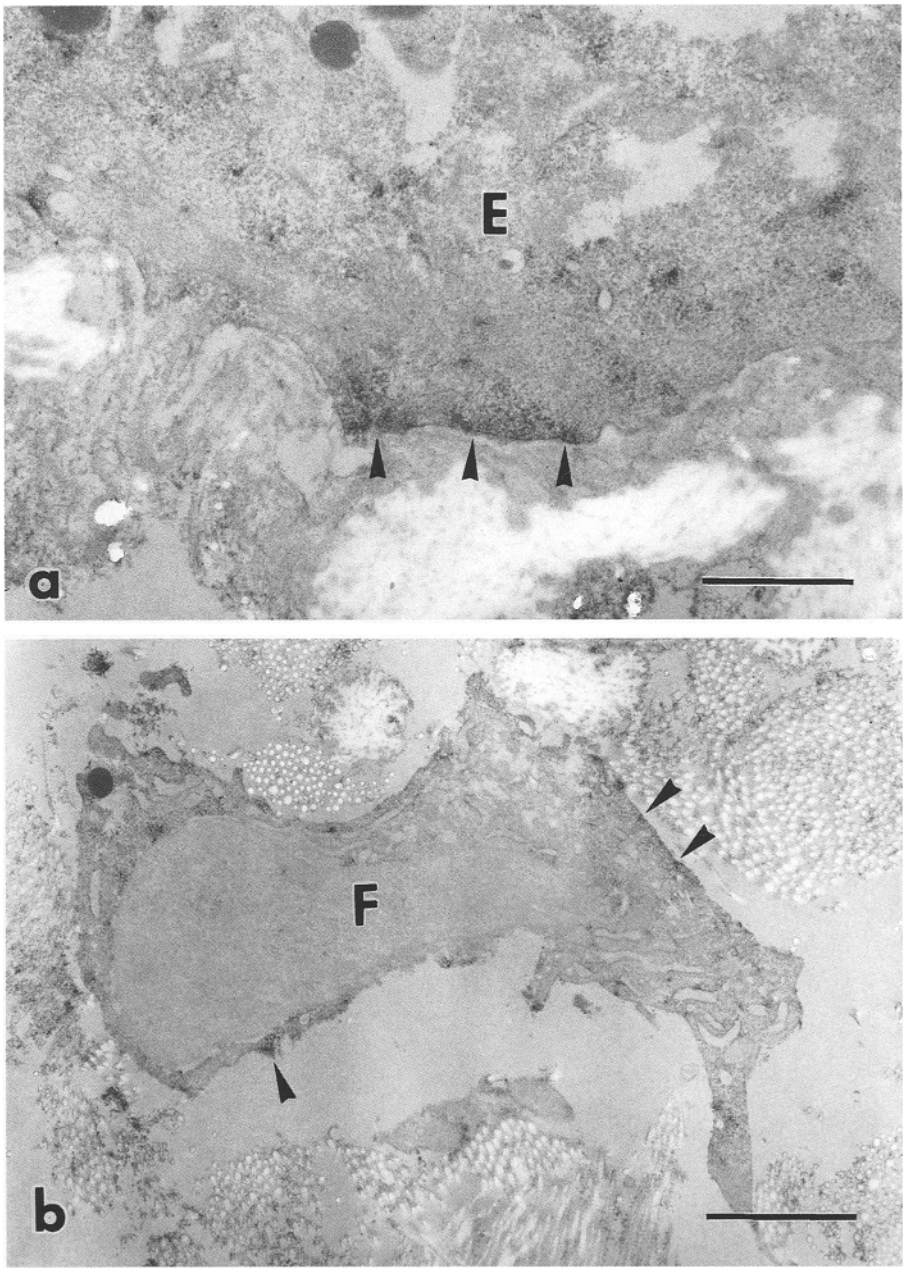


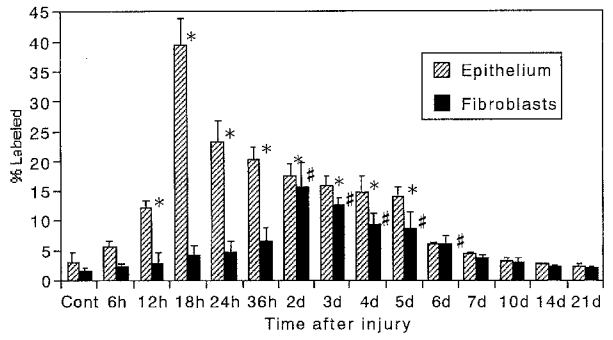
Fig. 7a, b Immunoelectron micrograph for vinculin (early stage). Two days after injury, vinculin is present on the basal surfaces, where actin filaments are assembled, of regenerating epithelial cells (*F*, *arrowheads*; **a**) and in a spotty pattern at the fibroblast peripheries (*F*, *arrowheads*; **b**). *Bars* **a** 1 μm, **b** 2 μm

coated filters demonstrated significant stimulation of bovine bronchial epithelial cell migration, but laminin- and type IV collagen-coated filters were less active [25]. Furthermore, we observed that both $\alpha 5\beta 1$ and vinculin appeared on the basal surface of the regeneration epithelial cells a little later than did plasma fibronectin. After corneal injury, a 12-h healing period was needed for the expression of $\beta 1$ integrins after injury [22] and the distribution of integrin receptors in vitro was found to be regulated by ligands occupancy [17]. In this context, we emphasize that fibronectin plays an important part in the induction of adhesion complex formation in the regenerating epithelial cells during wound healing.

During the early stage, no cellular fibronectin was detected using light microscopy, although fibronectin was observed in the rough endoplasmic reticulum of regenerating epithelial cells in the immunoelectron microscopic study, which suggests that the extracellularly deposited fibronectin originated from plasma fibronectin, although the regenerating epithelial cells were already producing fibronectin at this stage. Therefore, we consider that plasma fibronectin is far more important than cellular fibronectin for the initial epithelial regeneration process, although cellular fibronectin appears to be involved in the later stages.

After the wounded area had been completely covered with regenerating epithelium, $\alpha 5\beta 1$ was localized on the cell surfaces of the basal layer of the regenerating epithelium, which showed squamous metaplasia. The same distribution was observed during corneal wound healing, in epidermal explants [12] and in normal human skin [24].

a



b

	Cont	6h	12h	18h	24h	36h	2d	3d	4d	5d	6d	7d	10d	14d	21d
Fn	±	++	++	++	++	++	++	++	++	++	++	+	+	±	±
cFn	–	–	–	–	–	–	–	±	+	++	++	+	+	±	–
Epithelium															
α5β1	±	±	+	+	++	++	++	++	++	++	++	+	±	±	±
Vc	±	±	+	+	++	++	++	++	++	++	++	+	±	±	±
A	±	±	+	+	++	++	++	++	++	+	+	±	±	±	±
Fibroblasts															
α5β1	–	–	–	–	–	–	±	±	+	++	++	+	+	±	–
Vc	–	–	–	–	–	–	±	±	+	++	++	+	+	±	–
αSMA	–	–	–	–	–	–	–	±	+	+	+	+	±	±	–

Note: –; No reaction, ±; Weak reaction, +; Moderate reaction, + +; Strong reaction

Fig. 8 **a** The BrdU labeling index and **b** reactions for fibronectin (*Fn*), cellular fibronectin (*cFn*), integrin $\alpha5\beta1$ ($\alpha5\beta1$), vinculin (*Vc*), actin (*A*) and α-smooth muscle actin (αSMA) of regenerating epithelial cells and fibroblasts during the healing process. The regenerating epithelial cell labeling index differed significantly from the control value ($P<0.05$) from 12 h to 5 days after injury (*). The fibroblast labeling index differed significantly from the control value ($P<0.05$) from 2 to 6 days after injury (#). The Fn, cFn, $\alpha5\beta1$, Vc and A reactions of each cell type correlated well with the respective BrdU labelling index

Vinculin, however, was localized on the cell surfaces of all the regenerating tracheal epithelial layers. Vinculin is known to be associated with cadherin [20], which is a cell-cell adhesion molecule [32], and the vinculin distribution in all the regenerating epithelial layers we observed probably corresponds to the distribution of cadherin in the zonula adherens.

The distribution and appearance of fibronectin, $\alpha5\beta1$, vinculin and actin in the regenerating epithelium during the healing process were well coordinated, and these components seem to play a central part in the initial migration and settlement of regenerating epithelial cells after injury.

Plasma fibronectin was detected in the interstitium much earlier than cellular fibronectin. Therefore, we consider that plasma fibronectin is important for the induction of adhesion complex formation in fibroblasts, as it appeared to be in the regenerating epithelial cells.

Recent studies have demonstrated that fibroblasts are the major cells synthesizing cellular fibronectin during cutaneous wound healing in rats [4]. Cellular, but not plasma, fibronectin is required for collagen gel contraction by fibroblasts [2], and $\alpha5\beta1$ expression in fibroblasts precedes wound contraction during cutaneous would healing in pigs [34]. After tracheal injury, α-smooth muscle actin, $\alpha5\beta1$, vinculin and cellular fibronectin appeared in fibroblasts later than in regenerating epithelial cells and coincided approximately with the period of granulation tissue formation. These findings indicate that fibroblasts differentiate into myofibroblasts during granulation, produce cellular fibronectin, adhere tightly to extracellular components and are involved in wound contraction during the later stages of healing.

A previous study demonstrated that $\alpha5\beta1$ co-localized with vinculin and actin in cultured astrocytes [33]. In fibroblasts, co-alignment between actin filaments at the cell surface and extracellular fibronectin in vitro was demonstrated electron microscopically [7, 15, 28]. Our study showed co-localization of actin, vinculin, $\alpha5\beta1$ and fibronectin in fibroblasts in granulation tissue similar to that in regenerating epithelial cells during tracheal wound healing.

Integrin $\alpha5\beta1$ and vinculin appeared in both epithelial cells and fibroblasts during the most active stage of cell proliferation. Fibronectin is known to affect proliferation of epithelial cells by mediating their anchorage [30], to inhibit the terminal differentiation of human keratinocytes [1] and to function as a growth factor for pulmonary fibroblasts [3]. In this study, cellular fibronectin appeared in epithelial cells and fibroblasts a little later than the peak BrdU labeling index was observed. Consequently, our results suggest that plasma fibronectin is more important than cellular fibronectin for cell proliferation, that regenerating epithelial cells migrate into the wound before they proliferate, and that the adhesion molecule $\alpha5\beta1$ is involved in proliferation of both regenerating epithelial cells and fibroblasts by mediating their anchorage, as well as cell migration and settlement.

We conclude that fibronectin, $\alpha5\beta1$, vinculin and actin appear in regenerating epithelial cells virtually simultaneously after tracheal mucosal injury and that cellular fibronectin, $\alpha5\beta1$, vinculin and α-smooth muscle actin appear virtually simultaneously in fibroblasts. These components appear in regenerating epithelium earlier than in fibroblasts, and the immunostaining reactions for fibronectin, $\alpha5\beta1$ and vinculin in the regenerating epithelial cells and fibroblasts correlate well with their BrdU labelling indices. Plasma fibronectin and $\alpha5\beta1$ may stimulate cell migration and proliferation during the early stages of tracheal wound healing before cellular fibronectin is produced in situ.

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