Fibronectin Promotes Epithelial Migration of Cultured Rabbit Cornea In Situ

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ABSTRACT We investigated the effect of fibronectin on epithelial migration onto the stroma in cultured rabbit cornea. Rabbit plasma fibronectin was purified by affinity chromatography using gelatin-Sepharose 4B, and its purity was confirmed by SDS polyacrylamide slab gel electrophoresis. Antibody against rabbit plasma fibronectin raised in guinea pigs formed a single precipitin line against rabbit plasma and purified rabbit plasma fibronectin by Ouchterlony double diffusion test. When rabbit cornea was cut into small blocks and cultured in TCM-199 medium alone, corneal epithelial cells began to migrate on the cut edge of the corneal stroma. The addition of purified rabbit plasma fibronectin to the culture medium significantly enhanced epithelial migration. The degree of enhancement depended on the amount of fibronectin added. When guinea pig IgG anti-rabbit plasma fibronectin was added, epithelial migration was significantly inhibited when compared with that in control cultured corneal blocks. The results demonstrate that fibronectin promotes epithelial migration in the cornea and thus plays an important role in corneal wound healing.

Studies of the biological activities of fibronectin show that it is responsible for cell-to-cell and cell-to-matrix adhesion and cell spreading, and that it has opsonic activity (1–4). These biological activities have been shown primarily in cultured cells. For an understanding of the molecular mechanism of biological activities of fibronectin, the molecular structure of fibronectin has been extensively studied (5, 6). However, the physiological role or significance of fibronectin in situ has not yet been clarified.

Recently, morphological studies by immunofluorescence microscopy revealed the presence of fibronectin at the wound site. When corneal epithelial cells were removed mechanically, fibronectin specific fluorescence was observed beneath the regenerating epithelial cells (7). When a nonpenetrating knife cut was given to the rabbit cornea, fibronectin could be observed at the cut edge of the corneal stroma. When epithelial cells migrated, fibronectin appeared under the cells (8). Furthermore, Grinnell and associates reported that fibronectin appears at the wound site of guinea pig trunk skin (9).

Based on morphological studies and evidence that the fibronectin molecule binds to collagen, fibrinogen/fibrin, the involvement of fibronectin in wound healing has been strongly suggested (10, 11). However, there is no direct evidence of the effects of fibronectin on corneal wound healing.

When the cornea is damaged, the first step in wound healing is coverage of the wounded area by epithelial cells. Histological studies have shown that surrounding epithelial cells slide into the wounded area, and mitosis or cell proliferation follows from 24 to 30 h after the injury (12).

To investigate the role of fibronectin in corneal wound healing, we examined the effect of fibronectin on the migration of epithelial cells. For this, we cultured a block of rabbit cornea in serum-free medium and examined the epithelial migration on the corneal stroma as a model for corneal wound healing in vitro. To examine the specificity of the effect of fibronectin on epithelial migration, we added guinea pig IgG anti-rabbit plasma fibronectin to the medium and examined the changes of the rat of epithelial migration.

MATERIALS AND METHODS

New Zealand albino rabbits were purchased from Hamaguchi Animals (Osaka, Japan). Guinea pigs, Hartley strain, were obtained from Nihon Animals, Co. (Osaka, Japan). Gelatin-coupled Sepharose 4B and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Culture medium, TCM-199, and Hanks' balanced salt solution were obtained from Research Foundation for Microbial Disease of Osaka University (Suita, Japan). Freund's complete and incomplete adjuvant were purchased from Difco Laboratories (Detroit, MI). Bovine plasma albumin, crystallized, was obtained from Armour Phamaceutical Co. (Phoenix, AZ). Goat serum anti-guinea pig serum and goat serum anti-guinea pig IgG were purchased from Cappel Laboratories (Cochranville, PA). All other reagents were reagent grade.

Purification of Rabbit Plasma Fibronectin: Rabbit plasma fibronectin was purified according to the method of Engvall and Ruoslahti (13). Citrated rabbit whole blood was collected by heart puncture, and plasma was obtained by centrifugation at 3,000 rpm for 30 min at room temperature. Plasma was applied to the gelatin-coupled Sepharose 4B column. Extensive

washing was done with PBS containing 10 mM sodium citrate until no absorbance was detected at 280 nm by a UV monitor (Single path monitor, UV-1, Pharmacia Fine Chemicals). Bound fibronectin was eluated by 4 M urea in 0.05 M Tris-HCl buffer, pH 7.5. The eluated fibronectin was then dialyzed against PBS for 12 h at 4°C with five changes of PBS.

Preparation of Guinea Pig IgG Anti-rabbit Plasma Fibronectin: Purified rabbit plasma fibronectin (200 μg per animal) was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly into guinea pigs for primary immunization. Weekly booster immunizations of purified rabbit plasma fibronectin (200 μg per animal) emulsified with Freund's incomplete adjuvant were then given four times. 1 wk after the last immunization, guinea pig blood was collected by heart puncture, and serum was prepared by centrifugation at 3,000 rpm for 30 min at 4°C. Guinea pig serum was fractionated by 47% of saturated ammonium sulfate. Precipitate was dissolved in PBS, pH 7.2, and then dialyzed against 0.0175 M sodium phosphate buffer, pH 6.8. IgG fraction was collected from DEAE-sephacel column chromatography using 0.0175 M sodium phosphate buffer, pH 6.8.

SDS PAGE, Ouchterlony Double Diffusion Test, and Protein Determination: The purity of fibronectin was analyzed by SDS PAGE according to the method of Laemmli (14). The purity of guinea pig IgG anti-rabbit plasma fibronectin and of normal guinea pig IgG was analyzed by the Ouchterlony double diffusion test with 1% agarose gel in PBS. Protein determination was performed according to Lowry's method (15) with crystallized bovine plasma albumin as a standard.

Organ Culture of Rabbit Cornea: Organ culture of the rabbit cornea was carried out according to the method of Cameron (16). Rabbits were anesthetized with sodium pentobarbital (25 mg/kg body weight) before each eye was aseptically enucleated. The sclerocorneal section was excised, washed with Hanks' balanced salt solution, and cut into six blocks (2 × 4 mm) with a razor blade. Preliminary experiments showed that the size of the corneal block did not affect the rate of epithelial migration. Each corneal block was cultured separately in a tissue culture plate with 24 wells (Nunclon multidish 24 wells, Nunc, Roskilde, Denmark) with TCM-199 medium, with or without purified rabbit plasma fibronectin, guinea pig IgG anti-rabbit fibronectin, or normal guinea pig IgG. Each treatment group contained five blocks. To avoid the individual-to-individual variation, only one of the blocks from one rabbit was cultured in each treatment group, so that five blocks of each treatment group originated from five rabbits. The culture was maintained in a humidified incubator with 5% CO_2 at 37°C.

Measurement of Epithelial Migration and Statistical Analysis: The length of the path of epithelial migration was measured as described previously (17). Briefly, at a certain period of incubation, the corneal blocks were fixed with a mixture of 100% ethanol and glacial acetic acid (95:5) at room temperature for 2 h. Fixed materials were embedded in paraffin after immersion in xylene and paraffin. The blocks were sectioned at 4 μm. The specimens were stained with hematoxylin-eosin and photographed with Kodak Tri-X film under a light microscope with the aid of an automatic exposure meter (Nikon Optiphot, Nikon, Tokyo, Japan). The length of epithelial migration was measured 250 μm apart at four points on both sides of each block. The average of the length at eight points was considered the length of the path of epithelial migration in one block. Throughout the present experiments, the variation of each measurement in one block did not exceed 15% in SEM. Our data are shown as the average and SEM of five blocks that were cultured simultaneously. Statistical analysis was done by the unpaired Student T test.

RESULTS

SDS polyacrylamide slab gel electrophoretic pattern of rabbit plasma fibronectin shows that unreduced purified plasma fibronectin had two bands at the molecular weights of 220,000 and 440,000. When fibronectin was reduced with 1% 2-mercaptoethanol and 1% SDS at 100°C for 5 min, a single band of fibronectin at 220,000 was observed, showing that our fibronectin preparation was pure. No residual plasma proteins were detected by SDS polyacrylamide slab gel electrophoresis.

The purity and specificity of guinea pig IgG anti-rabbit plasma fibronectin and normal guinea pig IgG were examined by the Ouchterlony double diffusion test. As shown in Fig. 1, both guinea pig IgG anti-rabbit plasma fibronectin (*A*) and normal guinea pig IgG (*B*) formed single precipitin lines

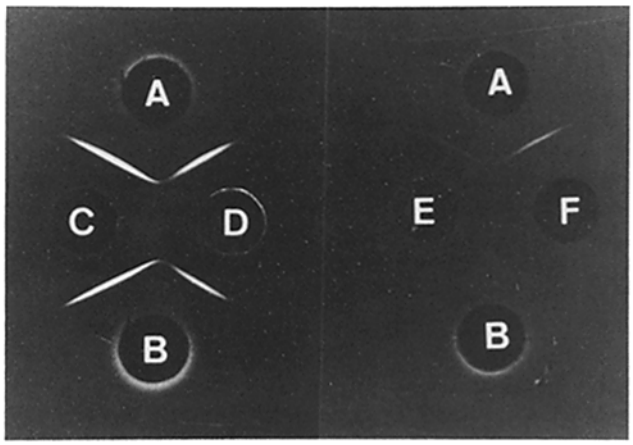

FIGURE 1 Ouchterlony double diffusion test on 1% agarose gel. *A*, guinea pig IgG anti-rabbit plasma fibronectin; *B*, normal guinea pig IgG; *C*, goat serum anti-guinea pig serum; *D*, goat serum anti-guinea pig IgG; *E*, rabbit plasma; *F*, purified rabbit plasma fibronectin.

against goat serum anti-guinea pig serum (*C*) and goat serum anti-guinea pig IgG (*D*). This showed that our guinea pig IgG was essentially pure. When the guinea pig IgG was reacted with rabbit plasma (*E*) or rabbit purified plasma fibronectin (*F*), guinea pig IgG anti-rabbit plasma fibronectin formed a single precipitin line against both rabbit plasma and fibronectin. No precipitin was observed between normal guinea pig IgG and rabbit plasma or fibronectin. These observations confirmed that guinea pig IgG anti-rabbit plasma fibronectin reacted with rabbit plasma fibronectin and that no cross-reactivity was observed, whereas normal guinea pig IgG did not react with rabbit plasma fibronectin.

Fig. 2 shows the histology of the cultured corneal block after 24 h. Epithelial cells migrated downward on the cut edge of the corneal block to the endothelial side.

We examined the dose response of rabbit plasma fibronectin on the migration of rabbit corneal epithelial cells. As shown in Table I, the length of the path of epithelial cells at 30 h after the initiation of organ culture increased with the increase of fibronectin added. When 60 μg/ml of fibronectin was added, the length of epithelial migration was 749 ± 28 μg, whereas that of control cultured blocks with medium alone was 578 ± 11 μm. The difference is statistically significant ($p < 0.001$). We then examined the time course of the effect of fibronectin on the length of the path of epithelial migration. As shown in Table II, the migration began gradually after the initiation of the culture. In the control group, the epithelial migration became prominent after 16 h, and the length of the path was 654 ± 50 μm at 32 h. The fibronectin-added (60 μg/ml) group, however, showed more rapid migration, and the length of the path was 942 ± 18 μm at 32 h. Statistical analysis shows that the difference of the length of the path of epithelial migration between the fibronectin-added group and the control group was significant at 24 h and 32 h after initiation of the culture ($p < 0.02$ and $p < 0.001$, respectively).

To examine the specificity of the effect of purified rabbit plasma fibronectin on the epithelial migration, we added guinea pig IgG anti-rabbit plasma fibronectin or unimmunized normal guinea pig IgG to the culture medium. As shown in Table III, no statistically significant difference was observed at any concentration of normal guinea pig IgG examined.

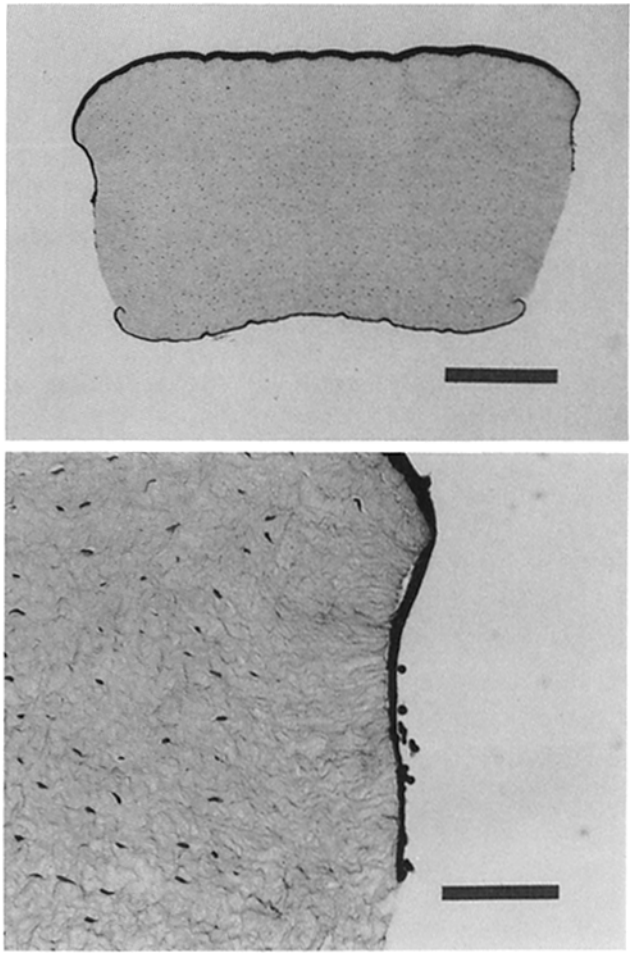

FIGURE 2 H-E staining of cultured corneal block after 24 h. Bar, 0.5 mm (*a*); 0.1 mm (*b*).

When IgG anti-rabbit fibronectin was added, however, the epithelial migration was suppressed significantly at the concentration of 250 and 1,000 μg/ml ($p < 0.005$ and $p < 0.001$, respectively). This was further confirmed by the time course experiment (Table IV). When anti-rabbit fibronectin was added at the concentration of 250 μg/ml, the epithelial migration was suppressed after 24 h. Compared with the control group, the difference was statistically significant at 24 h and at 32 h ($p < 0.02$ and $p < 0.001$, respectively).

DISCUSSION

These results clearly demonstrate that the addition of purified fibronectin promotes epithelial migration in cultured cornea. When rabbit corneal blocks were cultured in medium alone, epithelial migration occurred (Fig. 2). As we reported previously, fibronectin presents between the migrating epithelial cells and the collagen layer of the cornea (17). Fibronectin at this site might be involved in epithelial adhesion to the corneal stroma. Our immunohistochemical studies in several different injuries to the cornea, such as incision, experimental bullous keratopathy, and thermal burn, suggest that fibronectin might be synthesized by keratocytes in the corneal stroma (8, 18, 19).

Thus, newly synthesized fibronectin accumulates at the cut edge of the corneal block, and epithelial cells migrate on the coated fibronectin. When exogenous fibronectin was added to the culture medium, the epithelial migration was further enhanced. This evidence clearly demonstrates that fibronectin facilitates corneal epithelial migration in situ. This function of fibronectin was suppressed by the addition of IgG anti-rabbit plasma fibronectin, whereas the addition of unimmunized guinea pig IgG showed no effect on epithelial migration (Table III). These results show that the fibronectin molecule has specific activity to facilitate epithelial migration in the

TABLE I

Effect of the Amount of Added Purified Rabbit Plasma Fibronectin on Epithelial Migration at 30 h after the Initiation of Culture

FN treatment	Length of epithelial migration of five blocks					Mean ± SEM	*P* value
μg/ml			*μm*				
None	580	610	563	548	590	578 ± 10.7	
0.6	589	653	509	678	725	631 ± 37.5	NS
6.0	606	603	660	578	741	638 ± 29.1	NS
60.0	804	714	655	772	799	749 ± 28.4	$P < 0.001$

FN, purified rabbit plasma fibronectin; *NS*, not significant.

TABLE II

Time Course of Epithelial Migration in Cultured Rabbit Cornea with Medium Alone and with 60 μg/ml of Purified Rabbit Plasma Fibronectin

FN treatment	Incubation period	Length of epithelial migration of five blocks					Mean ± SEM	*P* value
μg/ml	*h*			*μm*				
60	8	21	29	28	31	1	22 ± 5.5	
None	8	24	10	24	11	42	22 ± 5.8	NS
60	16	144	117	122	164	114	132 ± 9.5	
None	16	126	147	105	79	125	116 ± 11.5	NS
60	24	625	503	488	609	468	539 ± 32.6	
None	24	421	470	363	440	303	399 ± 29.8	$P < 0.02$
60	32	902	930	996	912	971	942 ± 17.9	
None	32	509	804	705	598	655	654 ± 49.6	$P < 0.001$

FN, purified rabbit plasma fibronectin; *NS*, not significant.

TABLE III

Effect of the Amount of Added Guinea Pig IgG Anti-rabbit Plasma Fibronectin and of Normal Guinea Pig IgG on Epithelial Migration at 30 h after Initiation of Culture

Treatment		Length of epithelial migration of five blocks					Mean ± SEM	*P* value
μg/ml				*μm*				
aFN	62.5	554	602	543	602	455	551 ± 26.9	
IgG	62.5	778	527	712	628	519	633 ± 50.8	NS
aFN	250.0	491	280	379	290	287	345 ± 40.7	
IgG	250.0	593	496	722	543	559	583 ± 38.2	$P < 0.005$
aFN	1000.0	92	71	147	151	161	124 ± 18.0	
IgG	1000.0	668	642	442	690	796	648 ± 57.7	$P < 0.001$

aFN, guinea pig IgG anti-rabbit plasma fibronectin; *IgG*, normal guinea pig IgG; *NS*, not significant.

TABLE IV

Time Course of Epithelial Migration in Cultured Rabbit Cornea with Medium Alone and with 250 μg/ml of Guinea Pig IgG Anti-rabbit Plasma Fibronectin

aFN treatment	Incubation period	Length of epithelial migration of five blocks					Mean ± SEM	*P* value
μg/ml	*h*			*μm*				
250	8	7	12	9	10	3	8 ± 1.5	
None	8	28	12	5	7	12	13 ± 4.0	NS
250	16	91	115	76	69	99	90 ± 8.2	
None	16	120	141	99	91	129	116 ± 9.3	NS
250	24	322	307	277	291	238	287 ± 14.4	
None	24	292	428	366	453	479	404 ± 33.6	$P < 0.02$
250	32	360	272	331	231	263	291 ± 23.6	
None	32	615	518	596	460	570	552 ± 28.2	$P < 0.001$

aFN, guinea pig IgG anti-rabbit plasma fibronectin; *NS*, not significant.

cornea. Further studies into the molecular mechanisms of fibronectin in epithelial migration are required.

Fibronectin has been reported to be a multifunctional glycoprotein. It binds to collagen, heparin, fibrin, and glycosaminoglycans (20). These binding abilities provide characteristic biological activity of fibronectin at the molecular level. In other words, the binding sites for several different molecules make fibronectin an adhesive protein.

As reported, fibronectin is involved in cell-to-cell or cell-to-matrix adhesion in cell culture systems (1, 2). On the other hand, cells such as fibroblasts, baby hamster kidney cells and Chinese hamster ovary cells have receptor sites for fibronectin on their cell surface, although the biochemical identification has not yet been made (21). Therefore the fibronectin molecule bound to the cell surface acts as glue between cell and cell or between cell and substratum. Furthermore, binding of fibronectin to the cell surface alters the cytoskeletal structure of cells (22) and thus may facilitate cell motility. Further investigations will be required to learn the precise mechanism of fibronectin on corneal epithelial motility.

Fibronectin has also been reported to have chemotactic activity (23). We cannot conclude at this point whether any one or all of these biological activities of fibronectin are responsible for enhancing corneal epithelial migration. Exogenously added fibronectin binds to stromal collagen and acts as a chemotactic agent. Corneal epithelial cells then might slide onto the fibronectin, which acts as a glue between epithelial cells and stroma.

Recently, we reported the appearance of fibronectin beneath the migrating epithelial cells in cultured rabbit cornea by immunofluorescence microscopy (17). The biochemical and cell biological evidence of the biological activities and molecular structure of fibronectin support the hypothesis that fibronectin between the migrating epithelial cells and the corneal stromal collagen layer plays a role in the adhesion of migrating epithelial cells and the stromal layer. Our present results, together with previous morphological results, demonstrate that fibronectin facilitates epithelial migration on collagen in the cultured cornea in situ.

Generally, fibronectin has been believed to mediate adhesion and spreading of mesenchymal cells, such as fibroblasts, but not of epithelial cells. Our present result is controversial with respect to those previously reported (24–26). Federgreen and Stenn (24) reported that fibronectin was not the substance that supports the spreading of dissociated cells and the outgrowth of epidermal cells on plastic in mouse skin. Laminin or epibolin has been reported to facilitate epithelial or epidermal cell attachment on plastic surfaces (26, 27). However, recently, several investigations revealed that fibronectin could enhance epithelial adhesion (27–32). This discrepancy was explained by the finding that epithelial cells could attach to plastic if the surface was coated with fibronectin or if fibronectin was added to the medium before the cells attached to the plastic, but, when epithelial cells were already attached to plastic surface, fibronectin did not act on the cells (27). All of these previously reported works were done with dissociated cells and plastic culture dishes. In the present study, we examined the migration of corneal epithelial cells on the stromal collagen in situ and not on plastic. Furthermore, the substratum has a biologically organized structure and contains keratocytes that are of mesenchymal origin. Adhesive glycoproteins, such as fibronectin, laminin or epibolin, may play an important role in cell-to-substratum interaction. But this activity might depend on the origin of the cells and on the conditions of the substratum. Our present experimental model is close to in vivo situation, although further investi-

gations are necessary to understand the biological significance of these glycoproteins in vivo.

One of the most important events in wound healing is the rapid coverage of the wound by epithelial cells. In corneal wounds, surrounding epithelial cells slide onto the wounded area during the first 20 to 24 h after wounding, and then cell proliferation heals the wound (12).

The appearance of fibronectin at the wound site has been reported in the cornea (7, 8, 18, 19) and in the skin (9). After mechanical debridement of corneal epithelial cells, fibronectin has been observed under regenerating epithelial cells (7). When a nonpenetrating knife cut was given to the rabbit cornea, fibronectin-specific fluorescence was detected beneath the sliding epithelial cells. When an epithelial plug was formed, which is the final stage of wound healing, fibronectin disappeared (8). These results suggest the positive involvement of fibronectin in wound healing.

Our present culture system might provide a suitable model for the study of corneal wound healing at the molecular level. We have found that a persistent epithelial defect of the human cornea was healed by the instillation of fibronectin eyedrops (33) prepared from the patient's plasma (34). We also found that fibronectin eyedrops accelerate the healing rate of corneal epithelium in rabbits after iodine-vapor removal of epithelial cells in vivo (35). The results presented in this paper show the effect of fibronectin on the migration of epithelial cells on collagen in the cornea, and thus provide strong scientific evidence for our new treatment for persistent corneal epithelial defects.

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