Effects of Steroid, Retinoid, and Protease Inhibitors on the Formation of Acantholysis Induced in Organ Culture of Skins from Patients with Benign Familial Chronic Pemphigus

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Skin explants from two lesional areas and four normal-appearing areas of four patients with benign familial chronic pemphigus (BFCP) were organ cultured with and without various reagents. After 24-h culturing of involved skin with medium only, dissociation of keratinocytes, which was also observed prior to culturing, was exacerbated, and the epidermis became edematous, with a large section detaching from the dermis. These phenomena were not suppressed even when betamethasone, retinol acetate, or camostat mesilate (serine protease inhibitor) was added to the medium. On the other hand, in the cultures of uninvolved skin explants with medium only, widened intercellular spaces were observed 24–48 h after initiation of culture, and dissociation of keratinocytes and acantholytic clefts became apparent after 72 h. Such culture-induced acantholysis was almost completely suppressed by the addition of betamethasone, but not suppressed by the addition of retinol acetate, EDTA, N-ethylmaleimaide, or pepstatin A. Camostat and SBTI incompletely suppressed the acantholysis. These findings suggest the possibility that steroid may reduce blistering and that an organ culture of non-lesional benign familial chronic pemphigus (BFCP) skin may be useful for clarifying the pathogenesis, as well as for discovering new drugs for the treatment of BFCP. Further experiments are required to clarify the role of serine proteases in the acantholysis in this disease. *J Invest Dermatol 97:644–648, 1991*

Benign familial chronic pemphigus (BFCP), originally reported by Hailey and Hailey in 1939 [1], is a rare hereditary vesiclobullous disease that is characterized by recurrent vesicle and bullae appearing predominantly on the skin of the neck, groin, and axillae. The most characteristic histologic features of BFCP are widespread acantholysis involving the lower and middle epidermis and the occasional presence of corps ronds and grain cells [2].

In previous papers, some authors have demonstrated experimentally induced acantholysis in uninvolved BFCP skin by topical application of various stimuli (mechanical, physical, toxic, allergic, and bacteriologic), and have postulated that acantholysis can be induced by various external factors even in normal-appearing skin [3,4]. However, this experimental model is no longer used for investigation, as skin lesions must be induced in vivo, on patients' skin.

On the other hand, the presence of an "epidermal cell dissociating factor" in lesional skin was reported in a study of co-cultured normal human skin and BFCP skin [5]. Unfortunately, however, subsequent reports concerning this finding have not yet appeared.

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Abbreviations:
BFCP: benign familial chronic pemphigus
DMEM: Dulbecco's modified Eagle's medium
EDTA: ethylenediamine tetra-acetic acid
NEM: N-ethylmaleimaide
SBTI: soybean trypsin inhibitor

In this study, we initially examined the histologic changes that occurred in skin explants from lesional and non-lesional BFCP skin during organ culture. We then examined the effects of various reagents on the spontaneously occurring changes that took place during culturing.

MATERIALS AND METHODS

Patients Four adult patients (two men and two women) were studied. Cases Y, T, and K were from the same family. (Table I).

Skin Samples Skin samples from two involved areas (cases Y and K) and three normal-appearing areas (cases Y, T, and K) were used. For use as controls, three normal human skin samples were obtained during surgery. All skin samples were transferred immediately after removal into Dulbecco's modified Eagle's medium with 100 units/ml penicillin G, 60 μg/ml kanamycin, and 20 mM Hepes buffer (DMEM), stored at 4°C and used for organ culture within 3 h after the biopsy.

Reagents Chemical reagents used include the following: 1) betamethasone (Sigma); 2) retinol acetate (Sigma); 3) camostat mesilate [6] — (N,N - dimethylcarbamoylmethyl - 4 - (4 - guanidinobenzoyloxy)-phenylacetate), a synthetic serine protease inhibitor used for the treatment of pancreatitis in Japan (provided by ONO pharmaceutical Co. Ltd.); 4) soybean trypsin inhibitor (SBTI) (Sigma), a serine protease inhibitor; 5) ethylenediamine tetra-acetic acid (EDTA) (Sigma), a metal protease inhibitor; 6) N-ethylmaleimaide (NEM) (Sigma), a thiol protease inhibitor; and 7) pepstatin A (Sigma), a carboxyl protease inhibitor.

Organ Culture Organ culture was carried out with slight modifications to our previously described method [7]. Briefly, biopsied skin samples (two lesional skins from cases Y and K, and three

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Table I. Summary of Patients

Case	Age (years)	Sex	Duration of Disease (years)
Y[a]	59	Female	19
T[a]	35	Male	10
K[a]	30	Male	4
E	53	Female	10

[a] Familial cases:

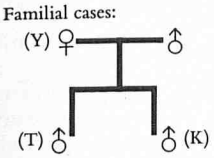

Table II. Spontaneously Occurring Changes and the Effect of Various Reagents on Such Changes in Organ Cultures of Involved BFCP Skin

Skin Sample		0 h	24 h	48 h	72 h
Case Y	DMEM only	2+[a]	3+[b]	4+[c]	4+
	Betamethasone		3+	4+	4+
	Retinol		3+	4+	4+
	Camostat		3+	4+	4+
Case K	DME only	2+	3+	4+	4+
	Betamethasone		3+	4+	4+
	Retinol		3+	4+	4+
	Camostat		3+	4+	4+

[a] 2+: Extended blister formation.
[b] 3+: Dissociation of keratinocytes, edema of epidermis, detachment of epidermis from dermis.
[c] 4+: 3+ and re-growth of remaining keratinocytes.

non-lesional skins from cases Y, T, and K) were carefully sliced and cut into small pieces (approximately 2 × 2 mm in size) in ice-cold DMEM. Approximately 30 pieces from each lesional skin sample and 50 pieces from each non-lesional skin sample were prepared. Six skin explants were then directly floated on 1.0 ml of DMEM with and without reagents. The final concentrations of each test material in the medium were 1) 100 μg/ml (0.25 mM) betamethasone, 2) 5 μg/ml (0.017 mM) retinol, 3) 2 mg/ml (4 mM) camostat, 4) 0.1 mM EDTA, 5) 0.1 mM NEM, and 6) 40 μg/ml (0.06 mM) pepstatin. Organ cultures were kept in humidified 5% CO_2-95% air at 37°C. Skin explants were harvested 24, 48, and 72 h after the cultures commenced. Consecutive H-E-stained sections were prepared from each skin explant and examined by light microscopy.

Using the methods outlined above, we evaluated the effects of different concentrations of betamethasone (0.25, 0.13, 0.06 mM), camostat (8.1, 4.0, 2.0 mM), or SBTI (0.23, 0.12, 0.06 mM) on the spontaneously occurring changes in the nonlesional skin explants from case E.

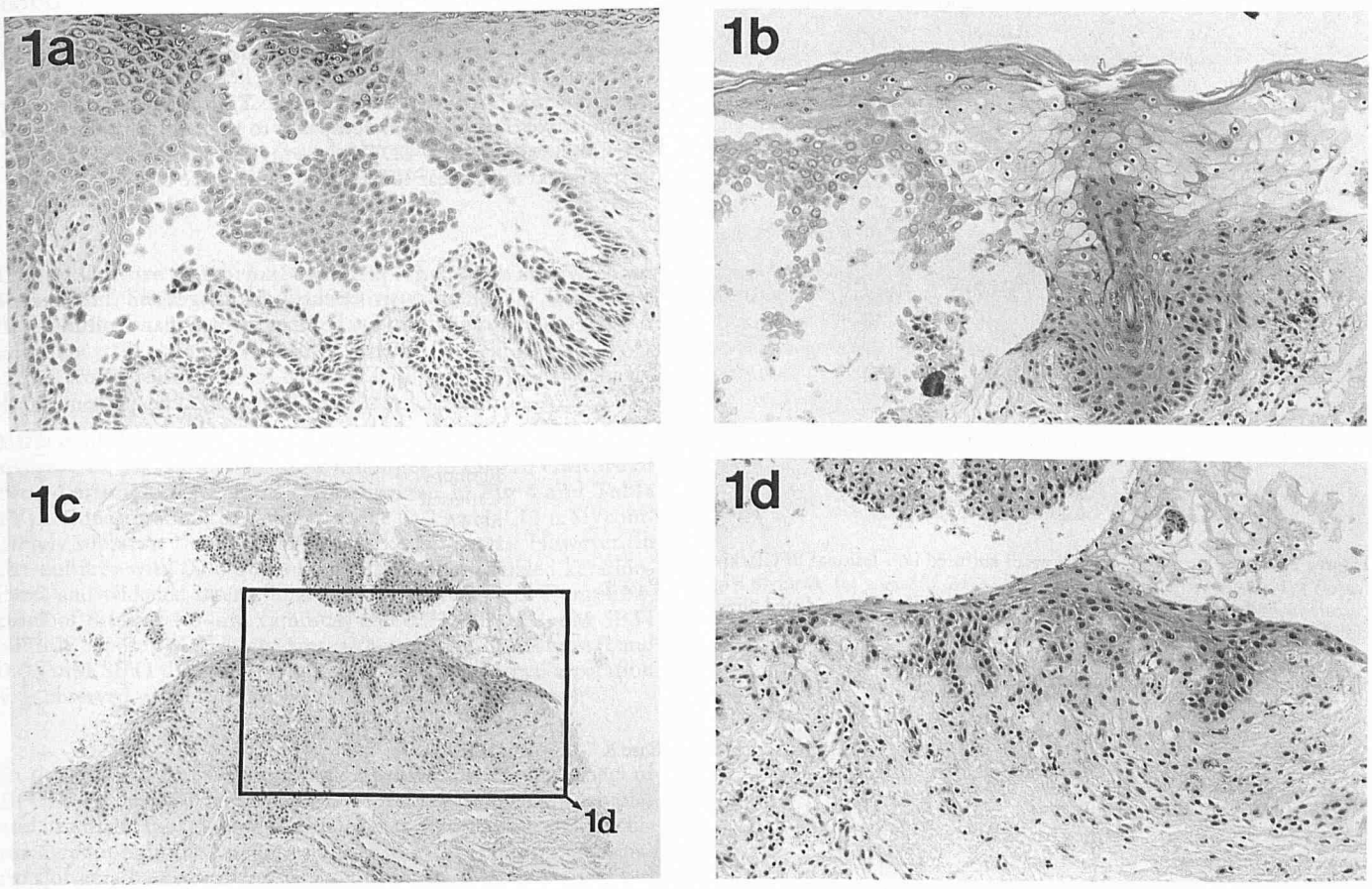

Figure 1. Observations of cultured lesional BFCP skin (case Y). As would be generally expected, acantholytic clefts were observed prior to culturing *(a)*. After 24-h culture, dissociation of epidermal cells was exacerbated, and the epidermis became edematous *(b)*. After 48–72 h of culture, these changes were exacerbated, and the remaining keratinocytes regrew *(c)*. *(d)* shows a higher magnification of indicating re-growth of keratinocytes. (*a, b,* and *d:* magnification ×160. *c:* magnification ×32.)

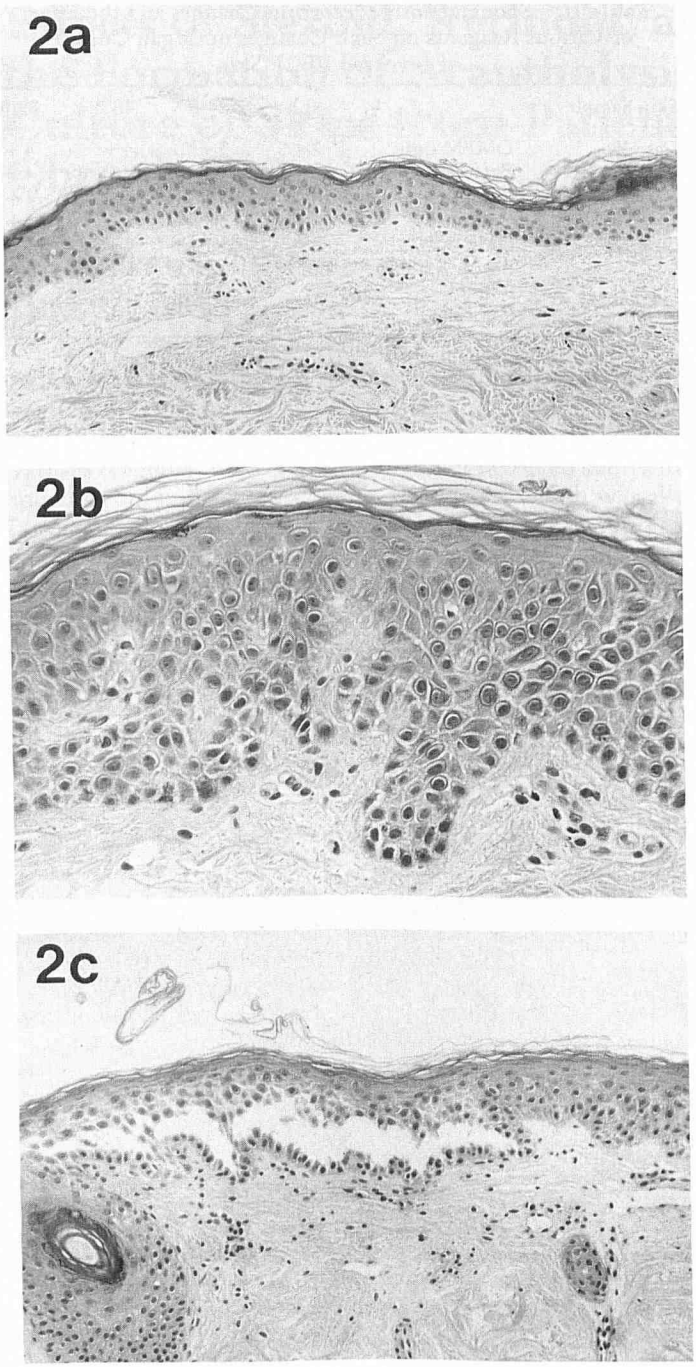

Figure 2. Representative observations of cultured non-lesional BFCP skin (case Y). There were no specific changes prior to culturing *(a)*. After 48 h of culture, rounded and eosinophilic keratinocytes, as well as a widened intercellular space, became apparent *(b)*. After 72 h of culture, dissociation of keratinocytes and epidermal cleft formation were observed. (*a* and *c:* magnification ×160. *b:* magnification ×320.)

RESULTS

Spontaneously Occurring Changes and the Effect of Various Reagents on Such Changes in Organ Culture of Involved BFCP Skin Samples Figure 1 demonstrates representative findings on lesional skin explants cultured with medium only. As would be generally expected, acantholytic clefts were observed in some areas of the biopsied skins prior to culturing (Figure 1*a*).

After 24 h of culture, dissociation of keratinocytes was exacerbated and the epidermis became edematous. A large section of the epidermis detached from the dermis (Fig 1*b*). After 48–72 h of culture, dissociation of keratinocytes and edematous changes in the epidermis increased (Fig 1*c*), and the remaining keratinocytes regrew (Fig 1*d*). Of note, re-growth of the remaining keratinocytes was observed in all skin explants cultured for 48–72 h with and without reagents. None of the reagents (betamethasone, retinol, or camostat) had any suppressive effect on these changes (Table II).

Spontaneously Occurring Changes and the Effect of Various Reagents on Such Changes in Organ Culture of Non-Lesional Skin Samples of BFCP Representative observations of cultured uninvolved BFCP skin samples with medium only are shown in Fig 2 and Table III. The structure of the epidermis was almost normal (Fig 2*a*), with the exception of rounded and eosinophilic keratinocytes and the widened intercellular space observed in case T (Table III, case T). The rounded keratinocytes and a widened intercellular space became apparent at 24 h (Table III, cases T and K) to 48 h (case Y) (Fig 2*b*), and dissociation of keratinocytes and cleft formation were generated 48–72 h after the cultures commenced (Table III, all cases) (Fig 2*c*). These changes were specific to the non-lesional BFCP skin cultures, differing from the damage or deterioration of the epidermis (such as vacuolation or dyskeratosis of keratinocytes) sometimes observed in the culture of skins from healthy donors.

The effects of various reagents on the spontaneously occurring changes in the organ cultures of uninvolved BFCP skins are summarized in Fig 3 and Table III. Among the various reagents, only betamethasone suppressed dissociation of keratinocytes in all cases through 72 h of culturing (Fig 3*b*), with the exception of rounded keratinocytes and a widened intercellular space observed in case T. On the other hand, retinol did not suppress such changes in any of the cases (Fig 3*d*). Culture with camostat suppressed the dissociation of keratinocytes in cases Y and K up until 72 h; thereafter rounded keratinocytes and widened intercellular spaces were observed (Fig 3*c*). Furthermore, apparent dissociation of keratinocytes and epidermal cleft formation were observed in case T, although such changes were relatively minor when compared to the control. Other protease inhibitors, such as EDTA, N-ethylmaleimaide (NEM), and pepstatin, did not appear to have any suppressive effect.

Table III. Spontaneously Occurring Changes and the Effect of Various Reagents on Such Changes in Organ Cultures of Uninvolved BFCP Skin

Skin Sample		0 h	24 h	48 h	72 h
Case Y	DMEM only	−[a]	−	+[b]	+
	Betamethasone		−	−	−
	Retinol		−	±[c]	+
	Camostat		−	−	±
	EDTA		−	+	+
	NEM		−	+	+
	Pepstatin		−	−	+
Case T	DMEM only	±	±	+	2+[d]
	Betamethasone		±	±	±
	Retinol		±	+	2+
	Camostat		±	+	+
	EDTA		±	+	2+
	NEM		±	+	2+
	Pepstatin		±	+	+
Case K	DMEM only	−	±	+	+
	Betamethasone		−	−	−
	Retinol		±	+	+
	Camostat		±	±	±
	EDTA		−	+	+
	NEM		±	+	+
	Pepstatin		±	+	+

[a] −: None.
[b] +: Apparent acantholysis.
[c] ±: Rounded keratinocytes, widened intercellular space.
[d] 2+: Extended blister formation.

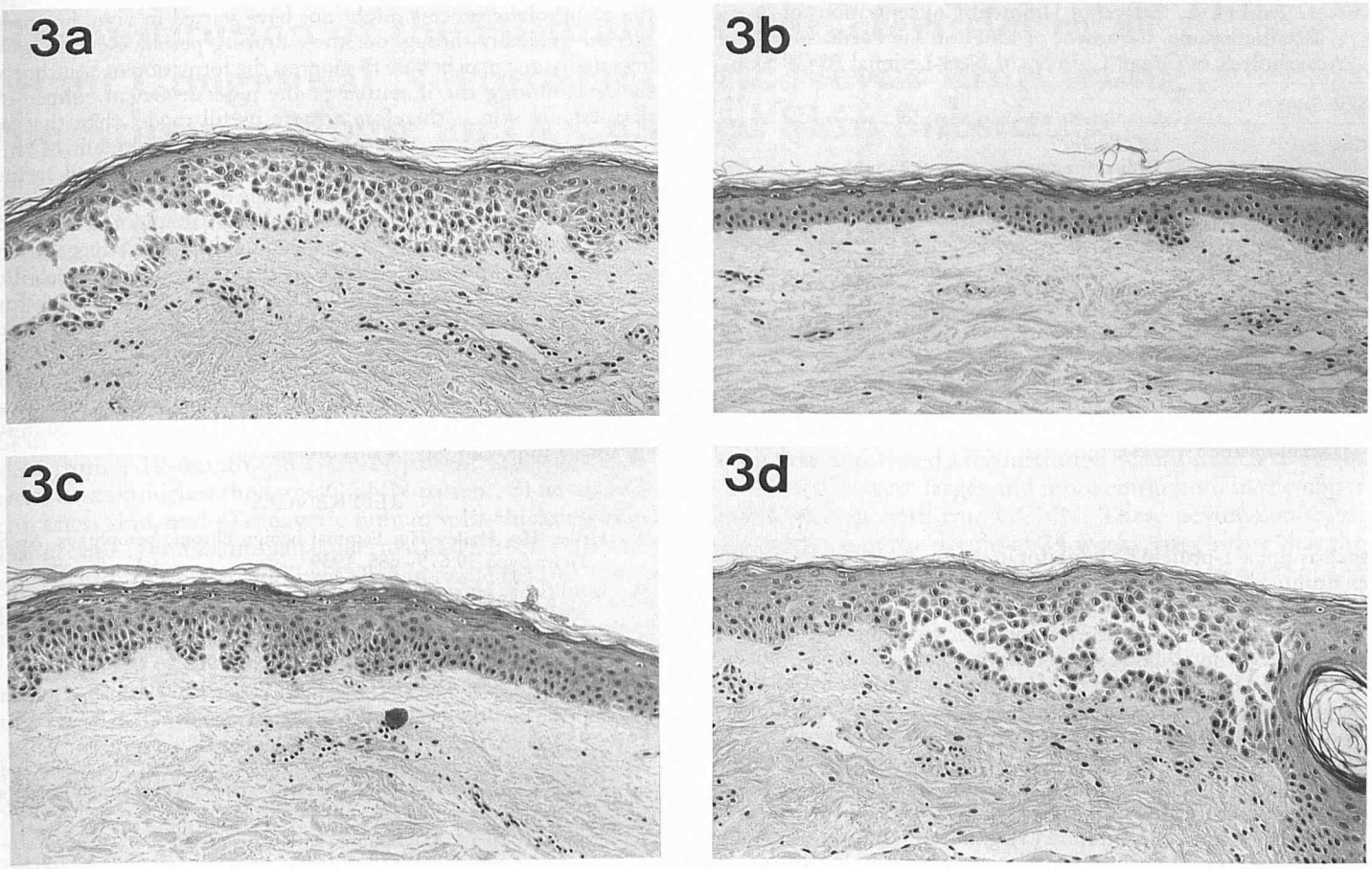

Figure 3. Observations of 72-h cultured non-lesional BFCP skin with and without reagents (case K). When the explants were cultured with medium only, dissociation of keratinocytes and formation of epidermal clefts were generated *(a)*. Such changes were inhibited by the addition of betamethasone *(b)*, and slightly suppressed by camostat. Rounded keratinocytes and a widened intercellular space were observed in the culture with camostat *(c)*. Other reagents such as retinol, EDTA, NEM, and pepstatin appeared not to have any suppressive effect *(d)*. (Magnification ×160.)

Organ Culture of Normal Skin Explants With and Without Reagents There were no specific histologic changes except for a deterioration in the epidermis (such as vacuolation or dyskeratosis of keratinocytes) observed in some of the normal skin explants obtained from healthy donors, cultured with and without the above-mentioned reagents (data not shown).

Effects of Different Concentrations of Betamethasone, Camostat, or SBTI on Histologic Changes in Organ Culture of Non-Lesional BFCP Skin (Summarized in Fig 4 and Table IV) Concentrations of betamethasone (0.25 and 0.13 mM) completely suppressed the dissociation of keratinocytes. However, in the cultures with 0.06 mM of betamethasone, rounded keratinocytes and widened intercellular space were observed. Concentrations of 8.1 and 4.0 mM camostat, and 0.23 and 0.12 mM SBTI slightly suppressed such changes, although 2.0 mM camostat and 0.06 mM SBTI did not. In addition, dermal-epidermal separation was observed in the culture with 8.1 mM camostat (Fig 4).

DISCUSSION

In this study, in attempting to establish an experimental model of BFCP, we initiated organ cultures of lesional BFCP skin samples and examined the histologic changes that occurred during the culturing period. In the case of lesional BFCP skin cultures, dissociation of keratinocytes increased in a time-dependent manner, and was not inhibited by the reagents tested. Therefore, we subsequently cultured skin explants from normal-appearing skin of patients with BFCP. Results showed that dissociation of keratinocytes occurred during cultivation, whereas the epidermal structure had appeared almost normal prior to culturing. Although the exact reason for this dissociation of keratinocytes in organ culture remains obscure, we speculate that the BFCP patients' skin samples may have been genetically abnormal, including even those samples from normal-appearing skin, and that direct mechanical stimuli during the preparation of explants and the elimination of inhibitory factors

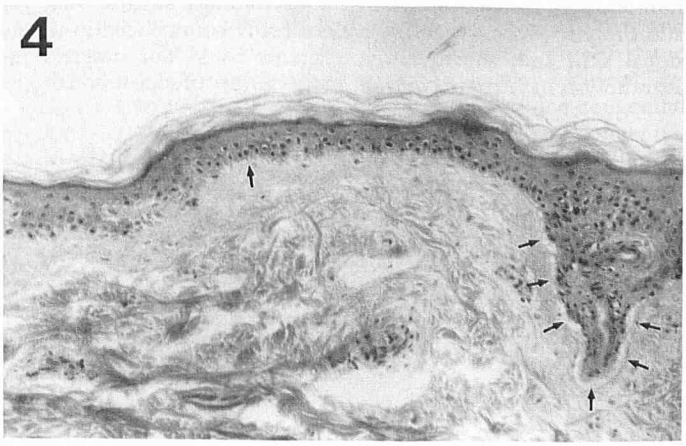

Figure 4. Non-lesional BFCP skin explant cultured with 8.1 mM camostat for 72 h. Whereas the dissociation of keratinocytes was suppressed, dermal-epidermal separation *(arrow)* was observed. (Magnification ×160.)

Table IV. Effects of Different Concentrations of Betamethasone, Camostat, or SBTI on the Formation of Acantholysis in Organ Cultures of Non-Lesional BFCP Skin

Skin Sample			72 h
Case E	DME only		+[c]
	Betamethasone	0.25 mM	−[a]
		0.13 mM	−
		0.06 mM	±[b]
	Camostat	8.1 mM	±[d]
		4.0 mM	±
		2.0 mM	+
	SBTI	0.23 mM	±
		0.12 mM	±
		0.06 mM	+

[a] −: None.
[b] ±: Rounded keratinocytes, widened intercellular space.
[c] +: Apparent acantholysis.
[d] Dermal-epidermal separation.

such as alpha-1-proteinase inhibitor in serum, as well as an increased environmental temperature, might be responsible for inducing such changes.

These spontaneously occurring changes were almost completely inhibited by the addition of 100 μg/ml betamethasone, but not inhibited by the addition of 5 μg/ml retinol. Various authors have suggested that the destruction of the desmosome tonofilament complex bound to the cell membrane is an initial event in the acantholytic phenomenon in BFCP [4,9,10], and have reported that 5 μg/ml retinol has a stabilizing effect on the cell membrane in a suspension culture of keratinocytes [8]. We selected, for this investigation, the same concentration (5 μg/ml) of retinol to attempt to stabilize cell membranes and inhibit acantholysis. However, this concentration of retinol failed to inhibit the dissociation of keratinocytes. The above-mentioned findings may be able to explain the effect of these drugs in vivo; strong topical steroids can reduce the formation of blisters, whereas the effect of retinoids is variable.

From among the various kinds of protease inhibitors utilized, serine protease inhibitors were able to suppress the dissociation of keratinocytes at higher concentrations, although the inhibitory effect appeared to be incomplete. These findings are consistent with the possible involvement of serine proteases in the process of acantholysis in BFCP. However, further experiments such as non-lesional skin cultures with the presence of fetal calf serum and/or human serum are required to clarify the exact role of serine proteases in this disease.

It is of interest to note that Jensen et al reported an increased level of plasminogen activator (a serine protease) in lesional, but not in non-lesional, BFCP skins [11]. Our findings suggest that the acantholytic process may have had already commenced in the lesional skin and, subsequently, reagents could not suppress the acantholysis in vitro. In contrast, in the culture of non-lesional skin, the acantholytic process might not have started in vivo, but progressed gradually throughout the culturing period. Accordingly, betamethasone may be able to suppress the formation of acantholysis by inhibiting the initiation of the process. Organ culture of non-lesional skin is therefore a more useful model than that of lesional skin in discovering new drugs for the treatment of this disease. The inhibitory mechanism of betamethasone and serine protease inhibitors, and the reasons for the differing effects of these reagents on lesional versus non-lesional skins are unknown. However, we suggest that a lag time is required for these reagents to diffuse into the epidermal cells, enter them, and inhibit metabolic events. In fact, the inhibitory effect of methylpredonosolone for pemphigus acantholysis in skin culture required 4–24 h lag time [12].

At present, we are investigating whether the culturing of normal skin explants with conditioned media can induce acantholysis. Results may provide us with information that will lead to understanding the pathophysiology of this disease.

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