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### Research Article

Fibroblast cultures from patients with dominant dystrophic epidermolysis bullosa of the albopapuloid variety display deranged glycosaminoglycan metabolism. These cells accumulate increased amounts of sulfated glycosaminoglycans. The mechanism for the greater content of glycosaminoglycans appears to be related to increased synthesis. During the first 6-12 h, intracellular labeled glycosaminoglycans accumulated in the dominant dystrophic epidermolysis bullosa cells at about twice the rate as that of control fibroblasts. In addition, secretion of sulfated glycosaminoglycans was two- to threefold greater than in control cultures. In contrast, both pulse-chase and cross-correction experiments failed to show any evidence for defective degradation of the material. The biochemical trait is genetically specific for albopapuloid dominant dystrophic epidermolysis bullosa, since fibroblasts from patients with other varieties of epidermolysis bullosa did not accumulate increased glycosaminoglycans. The data suggest that in vitro abnormality in glycosaminoglycan metabolism could serve as an important marker for this variety of epidermolysis bullosa and be of genetic and prognostic value in the sporadic patient with epidermolysis bullosa. Although the precise relationship of the defect to the disease has not yet been defined, it is possible that excessive tissue accumulation of glycosaminoglycans may alter collagen fibril deposition, thus, impairing the structural integrity of the skin and leading to posttraumatic blisters and erosions that characterize the disease.

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# Increased Glycosaminoglycan Accumulation as a Genetic Characteristic in Cell Cultures of One Variety of Dominant Dystrophic Epidermolysis Bullosa

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**ABSTRACT** Fibroblast cultures from patients with dominant dystrophic epidermolysis bullosa of the allopapuloid variety display deranged glycosaminoglycan metabolism. These cells accumulate increased amounts of sulfated glycosaminoglycans. The mechanism for the greater content of glycosaminoglycans appears to be related to increased synthesis. During the first 6–12 h, intracellular labeled glycosaminoglycans accumulated in the dominant dystrophic epidermolysis bullosa cells at about twice the rate as that of control fibroblasts. In addition, secretion of sulfated glycosaminoglycans was two- to threefold greater than in control cultures. In contrast, both pulse-chase and cross-correction experiments failed to show any evidence for defective degradation of the material. The biochemical trait is genetically specific for allopapuloid dominant dystrophic epidermolysis bullosa, since fibroblasts from patients with other varieties of epidermolysis bullosa did not accumulate increased glycosaminoglycans. The data suggest that the *in vitro* abnormality in glycosaminoglycan metabolism could serve as an important marker for this variety of epidermolysis bullosa and be of genetic and prognostic value in the sporadic patient with epidermolysis bullosa. Although the precise relationship of the defect to the disease has not yet been defined, it is possible that excessive tissue accumulation of glycosaminoglycans may alter collagen fibril deposition, thus, impairing the structural integrity of the skin and leading to posttraumatic blisters and erosions that characterize the disease.

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## INTRODUCTION

Epidermolysis bullosa (EB)<sup>1</sup> refers to a group of genetic disorders characterized by severe blistering after minor trauma to the skin (1). At least four major varieties of the disease exist—dominant dystrophic EB, dominant EB simplex, recessive dystrophic EB, and recessive EB letalis—and, although they differ from one another in the degree of severity, each is accompanied by profound morbidity as a result of repeated cutaneous blistering throughout life (2).

Two subvarieties of dominant dystrophic epidermolysis bullosa (DDEB) occur, the allopapuloid variety of Pasini (3) and the hyperplastic variety of Cockayne (4) and Touraine (5). Each has its onset at birth or in early infancy and can only be differentiated by the occurrence in the allopapuloid variety of small, flesh-colored papules primarily on the trunk near the time of puberty. These lesions, the so-called allopapuloid lesions, appear spontaneously in the absence of antecedent trauma (1).

The etiology of either form of DDEB is unknown. However, histochemical examination has revealed an increased amount of degraded chondroitin sulfates in the skin of a patient with allopapuloid DDEB (6). Furthermore, urinary excretion of both native and partially degraded chondroitin sulfate B was also increased in the patient (7), suggesting that allopapuloid DDEB might be a disorder resulting from abnormal catabolism of acid mucopolysaccharide (6).

If the allopapuloid variety of DDEB were characterized by an abnormality in glycosaminoglycan metabo-

<sup>1</sup> Abbreviations used in this paper: DDEB, dominant dystrophic epidermolysis bullosa; EB, epidermolysis bullosa.

lism, it might be expected that the aberration would be manifested in fibroblast cultures. In the present study we have examined glycosaminoglycan metabolism in fibroblast cultures of seven patients with this variety of DDEB to determine (a) whether deranged glycosaminoglycan metabolism characterizes this form of EB, (b) the nature of any such abnormality in glycosaminoglycan metabolism, and (c) whether this biochemical abnormality might be used as a genetic marker to differentiate albopapuloid DDEB from other varieties of the disease.

## METHODS

**Fibroblast cultures.** DDEB fibroblast cultures were established from a 3-mm skin punch biopsy after obtaining informed consent. All biopsies were taken from the normal-appearing skin of the upper extremities, and not from blistered areas or albopapuloid lesions. Control cultures were initiated from upper extremity skin of healthy volunteers or were purchased from the American Type Culture Collection, Rockville, Md. As a further control, fibroblast cultures were established from patients with other genetically distinct forms of EB, recessive dystrophic EB and dominant EB simplex. All patients were examined by at least one of us (Dr. Bauer) and classified based on clinical, genetic, and histologic findings as described previously (8). The cell lines employed in this study are detailed in Table I.

Cells were grown in plastic culture flasks (Corning Glass Works, Science Products Div., Corning, N. Y.) in Dulbecco's Modified Eagle's Medium-high glucose + glutamine with 10% dialyzed fetal calf serum and 200 U of penicillin and 200 µg of streptomycin/ml at 37°C in an air-CO<sub>2</sub> atmosphere (95–5%). The

culture medium was altered by substituting MgCl<sub>2</sub> for MgSO<sub>4</sub> and reducing the NaHCO<sub>3</sub> concentration to 1.6 g/liter (9, 10).

**Incorporation of <sup>35</sup>SO<sub>4</sub><sup>2-</sup> into glycosaminoglycans.** For labeling experiments, confluent cultures were washed three times and incubated in the above medium adjusted to pH 6.8 with nonvolatile buffers, 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) and 15 mM N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, as described by Lie et al. (10). The cells were labeled with 4–20 × 10<sup>6</sup> cpm/ml of carrier-free Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (New England Nuclear, Boston, Mass.) and at varying intervals, the cells were washed with Hanks' balanced salt solution, trypsinized, harvested by centrifugation, and extracted four times with boiling 80% ethanol as described by Fratantoni et al. (9). The extracted cell residue was dissolved in 10% NaOH for determination of radioactivity and cell protein. Labeled glycosaminoglycans were determined in the medium after dialysis for 4 h against 0.1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 48 h against running tap water before determining the radioactivity (9).

**Degradation of <sup>35</sup>SO<sub>4</sub><sup>2-</sup>-labeled glycosaminoglycans.** Degradation rates for [<sup>35</sup>SO<sub>4</sub><sup>2-</sup>]glycosaminoglycans were determined as described by Fratantoni et al. (9). Briefly, confluent cell cultures were prelabeled by incubation either for 24 or 72 h in the pH 6.8-adjusted medium with Na<sub>2</sub><sup>35</sup>SO<sub>4</sub>. The radioactive medium was then removed, the cells were passed and replated, and then incubated in the same pH-adjusted medium for specified time periods after which intracellular [<sup>35</sup>SO<sub>4</sub><sup>2-</sup>]glycosaminoglycans were determined as described above.

**Characterization of [<sup>35</sup>SO<sub>4</sub><sup>2-</sup>]glycosaminoglycans.** The molecular weight distribution of extra- and intracellular <sup>35</sup>SO<sub>4</sub><sup>2-</sup>-labeled material was determined using gel filtration (9). Samples were applied to a 1.6 × 35-cm column of Sephadex G-200, equilibrated with 0.02 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and then eluted at a flow rate of 10 ml/h.

**Uptake of <sup>35</sup>SO<sub>4</sub><sup>2-</sup>-labeled compounds.** <sup>35</sup>SO<sub>4</sub><sup>2-</sup>-labeled compounds to be used for the endocytosis studies were pre-

TABLE I  
Description of Human Skin Fibroblast Lines

Controls	Albopapuloid DDEB	Other DDEB	Recessive dystrophic EB	Dominant EB simplex
CRL 1187 (M 14)*	WUE 7562 (F 64)‡	WUE 76111 (M 20)	WUE 76108 (M 4)	WUE 7502 (F 19)
CRL 1119 (M 15)	WUE 7559 (F 21)‡	WUE 7512 (M 19) <sup>¶</sup>	WUE 7504 (F 8)	WUE 7535 (M 10)
WUN 76128 (F 47)	WUE 7570 (F 25)§	WUE 7513 (M 1) <sup>¶</sup>		
WUN 76130 (F 20)	WUE 7557 (M 21)§			
WUN 76135 (M 26)	WUE 7508 (F 10)			
WUN 77201 (M 25)	WUE 7560 (F 12)			
WUN 76131 (F 27)	WUE 77264 (F 25)			
CRL 1220 (M 15)				
CRL 1224 (F 40)				
CRL 1222 (M 8)				
WUN 7561 (M 33)				
WUN 7566 (F 30)				
WUN 77202 (F 23)				
WUN 77242 (F 19)				
WUN 77243 (F 30)				
WUN 77244 (M 30)				

\* Donor sex and age are given in parentheses.

‡ WUE 7562 and WUE 7559 are mother and daughter, respectively.

§ WUE 7570 and WUE 7557 are siblings.

¶ WUE 7512 and WUE 7513 are father and son, respectively.

pared by labeling confluent cultures of either normal or albopapuloid DDEB cultures for 72 h with  $^{35}\text{SO}_4^-$ . The medium was harvested and dialyzed at 4°C for 48 h against 0.1 M  $(\text{NH}_4)_2\text{SO}_4$  followed by dialysis for 48 h against 0.15 M NaCl (11). This material was sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.) before use.

Endocytosis of labeled material was determined by incubating confluent normal and DDEB cells at 37°C for 7 h at pH 6.8 in 2.0 ml of medium containing the  $^{35}\text{SO}_4^-$ -labeled compounds as described by Kresse et al. (12). Briefly, after incubating each of the cell types with either control-derived or DDEB-derived labeled material, the medium was removed and the cell layer was quickly washed five times with Hanks' balanced salt solution. The cells were then harvested by trypsinization and centrifugation and washed one additional time after which they were solubilized for determination of radioactivity and protein (12). Ethanol-soluble degradation products were determined in the culture medium by adding three volumes of ethanol and allowing the medium to stand at 25°C for at least 3 h. After centrifugation, the radioactivity of the supernate was determined (11, 12). Endocytosis was defined as the sum of the intracellular radioactivity plus the extracellular labeled ethanol-soluble material minus the ethanol-soluble radioactivity of a medium blank (i.e., the same amount of  $^{35}\text{SO}_4^-$ -labeled material added to 2 ml of conditioned medium from the same cell lines and incubated at 37°C for 7 h) (11). Blank values represented 2–4% of the total radioactivity added to the cultures. The mean recovery of radioactivity added to the cultures was 91.3% (range, 84–99%).

*Other assays.* Chemical quantitation of total hexuronic acid was determined by the carbazole method (13). Cells were lysed by sonication and a sample was removed for protein determination. The remainder of the lysates were hydrolyzed for 4 h at 37°C in a final concentration of 0.15 M NaOH. After neutralization with acetic acid, any insoluble residue was removed by centrifugation and the glycosaminoglycans were precipitated with 1% cetylpyridinium chloride. This precipitate was dissolved in 0.075 M NaCl and assayed. Protein was determined using established methods (14).

## RESULTS

The kinetics of incorporation of  $^{35}\text{SO}_4^-$  into intracellular glycosaminoglycans in three representative albopapuloid DDEB lines is depicted in Fig. 1. In normal cells, the amount of intracellular  $^{35}\text{SO}_4^-$  in glycosaminoglycans increased for 3–4 d after which a steady state was reached. In contrast, in the albopapuloid DDEB cell lines, radioactivity in intracellular glycosaminoglycans continued to increase for 6–8 d in one line and for up to 11 d, the longest time period examined, in two lines.

Rates of accumulation of intracellular  $^{35}\text{SO}_4^-$  glycosaminoglycans were also examined at early times in culture (Fig. 2). In control fibroblasts, incorporation of label into glycosaminoglycans was more rapid for the first 12–24 h, after which accumulation of  $^{35}\text{SO}_4^-$  glycosaminoglycans proceeded at a slower rate. Accumulation of intracellular  $^{35}\text{SO}_4^-$  glycosaminoglycans by the albopapuloid DDEB fibroblast lines occurred at a rate approximately twice that of the control cells for the first 6–12 h. It is noteworthy that when fibroblasts from other, genetically different types of EB were examined (Fig. 2A, WUE 76111 and WUE 7504; Fig. 2B, WUE

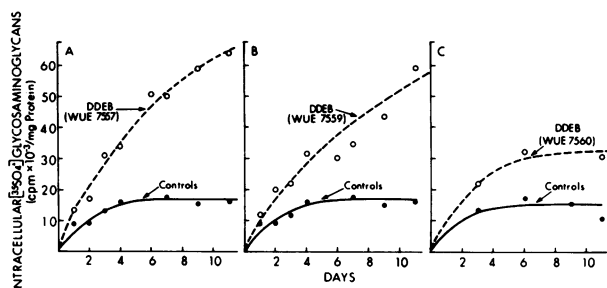


FIGURE 1 Incorporation of  $^{35}\text{SO}_4^-$  into intracellular glycosaminoglycans. Control and DDEB fibroblast cultures were incubated at pH 6.8 in the presence of  $^{35}\text{SO}_4^-$  as described in Methods. (A) WUE 7557 represents values from seven different experiments. (B) WUE 7559 represents values from nine experiments. (C) WUE 7560 depicts two different experiments. ●, control; ○, DDEB.

76108), they were found to accumulate  $^{35}\text{SO}_4^-$  glycosaminoglycans at rates equal to or slightly less than those of the control lines.

The increased accumulation of labeled glycosaminoglycans in albopapuloid DDEB cultures was also apparent in the culture medium. Fig. 3 depicts the kinetics of secretion of  $^{35}\text{SO}_4^-$  glycosaminoglycans in three different albopapuloid DDEB cell lines. Secretion was

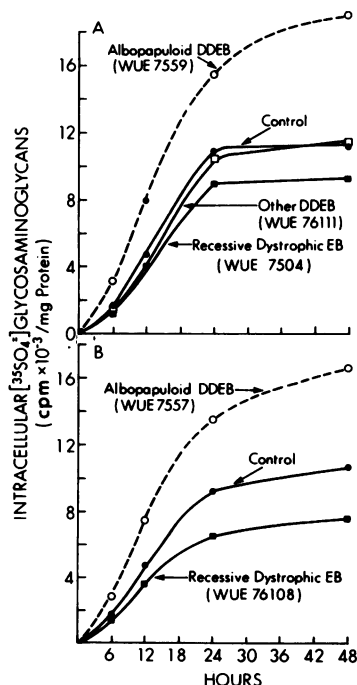


FIGURE 2 Short-term incorporation of  $^{35}\text{SO}_4^-$  into intracellular glycosaminoglycans. (A) Experiment 1: ●, control (CRL 1119); ○, albopapuloid DDEB (WUE 7559); □, other DDEB (WUE 76111); ■, recessive dystrophic EB (WUE 7504). (B) Experiment 2: ●, control (CRL 1222); ○, albopapuloid DDEB (WUE 7557); ■, recessive dystrophic EB (WUE 76108).

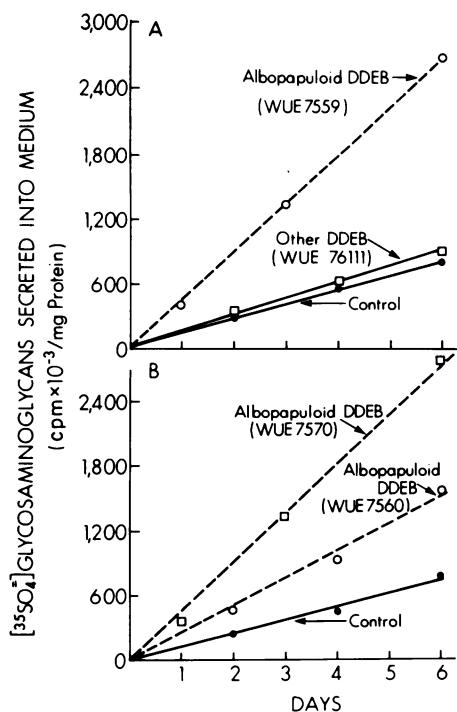


FIGURE 3 Secretion of [ $^{35}\text{S}$ ] $\text{SO}_4^-$  glycosaminoglycans into culture medium. The data are depicted as cumulative secretion into the medium. (A) Experiment 1: ●, control (WUN 76128); ○, albopapuloïd DDEB (WUE 7559); □, other DDEB (WUE 76111). (B) Experiment 2: ●, control (CRL 1224); □, albopapuloïd DDEB (WUE 7570); ○, albopapuloïd DDEB (WUE 7560).

linear for up to 6 d and was approximately threefold increased in two albopapuloïd DDEB cell lines (Fig. 3A, WUE 7559; Fig. 3B, WUE 7570) and about twofold greater in the third cell line (Fig. 3B, WUE 7560). Again, it is noteworthy that cells taken from a patient with a nonalbopapuloïd form of DDEB (WUE 76111) secreted [ $^{35}\text{S}$ ] $\text{SO}_4^-$  glycosaminoglycans at a rate equal to the control cultures (Fig. 3A).

In separate experiments, [ $^{35}\text{S}$ ] $\text{SO}_4^-$  glycosaminoglycan accumulation was determined in both the cells and the culture medium at a single time (Table II). As a group, the DDEB cells displayed approximately a threefold increase in intracellular labeled material (range, 1.4- to 5.4-fold;  $P < 0.01$ ), whereas there was a twofold increase in total labeled glycosaminoglycans (medium + cells) in these cultures (range, 1.2- to 2.3-fold;  $P < 0.005$ ).

To characterize the distribution of the [ $^{35}\text{S}$ ] $\text{SO}_4^-$  labeled material further, an additional experiment was performed in which the [ $^{35}\text{S}$ ] $\text{SO}_4^-$  labeled compounds were fractionated into intracellular material, cell layer-associated (pericellular) material released by trypsinization, and extracellular material found in the medium. In this experiment, at the end of the labeling period in the control cells, 11.3% of the labeled material was intra-

TABLE II  
[ $^{35}\text{S}$ ] $\text{SO}_4^-$  Glycosaminoglycans in Normal and Albopapuloïd DDEB Fibroblast Cultures

Culture	Number*	[ $^{35}\text{S}$ ] $\text{SO}_4^-$ Glycosaminoglycans, $\text{cpm} \times 10^{-3}/\text{mg protein} \dagger$	
		Intracellular	Total (medium + cells)
<b>Controls</b>			
WUN 76128	1	32.4	283.3
CRL 1224	1	38.1	373.8
WUN 76130	1	26.8	378.7
WUN 76135	1	43.8	277.6
Mean control		$35.3 \pm 3.7$	$328.4 \pm 27.7$
<b>DDEB</b>			
WUE 7559	4	$99.3 \pm 5.4$	$479.9 \pm 42.3$
WUE 7557	4	$145.6 \pm 3.7$	$645.9 \pm 20.0$
WUE 7562	4	$96.4 \pm 5.3$	$397.9 \pm 19.8$
WUE 7570	4	$189.3 \pm 5.7$	$721.6 \pm 31.2$
WUE 7508	4	$125.4 \pm 7.7$	$542.5 \pm 44.4$
WUE 77264	2	$47.7 \pm 1.0$	$701.8 \pm 13.8$
WUE 7560	1	65.8	740.7
Mean DDEB		$109.9 \pm 18.2 \S$	$604.3 \pm 50.1^{\parallel}$

\* Number of cultures examined in each cell line.

† Confluent cultures were incubated for 5 d with  $\approx 14 \times 10^6$  cpm/ml [ $^{35}\text{S}$ ] $\text{SO}_4^-$  after which intra- and extracellular glycosaminoglycans were determined. The extracellular [ $^{35}\text{S}$ ] $\text{SO}_4^-$  labeled compounds represent both secreted glycosaminoglycans and cell layer-associated labeled material released by trypsinization. Cell protein content varied from 0.70 to 0.97 mg/flask for the control lines and from 0.77 to 1.05 mg/flask for the DDEB lines. The data are expressed as mean  $\pm$  SE.

§  $P < 0.01$  with respect to the control cultures.

$^{\parallel} P < 0.005$  with respect to the control cultures.

cellular, 7.9% was found in the cell layer-associated fraction, and 80.8% was in the medium. Similarly, in the albopapuloïd DDEB cultures, 10.7% was intracellular, 7.4% was cell layer-associated, and 81.9% was extracellular.

The results of the labeling studies were further substantiated by chemical analysis. As shown in Table III, the hexuronic acid content of five albopapuloïd DDEB cell lines was compared with that of seven different normal control lines. Although there was variation among the five DDEB lines examined, the mean intracellular uronic acid content was 1.6-fold increased based on cell protein ( $P < 0.025$ ).

Removal of the labeled glycosaminoglycan occurs not only by secretion but also by degradation. To determine whether the increase in glycosaminoglycans in the albopapuloïd DDEB fibroblasts was the result of a decrease in the rate of degradation, the loss of labeled glycosaminoglycans from the intracellular pool was examined. For these studies, a single large culture was

TABLE III  
Hexuronic Acid Content of Normal and  
Albopapuloid DDEB Fibroblasts\*

Culture	Number†	Hexuronic acid‡ µg/mg protein
Controls		
WUN 77202	3	4.2±0.7
WUN 7561	2	4.1±0.1
WUN 76130	2	6.0±1.6
WUN 77244	1	4.0
WUN 77243	1	4.8
WUN 77242	1	4.4
WUN 76131	1	4.7
Mean control		4.6±0.2
DDEB		
WUE 7562	4	5.7±0.5
WUE 7559	5	6.4±0.7
WUE 7557	3	6.9±0.9
WUE 7508	2	12.6±7.4
WUE 7560	4	5.5±0.9
Mean DDEB		7.4±1.3 <sup>  </sup>

\* Confluent cultures of fibroblasts were harvested by trypsinization and the intracellular hexuronic acid was determined after precipitation of the glycosaminoglycans with 1% cetylpyridinium chloride.

† Number of cultures examined in each cell line.

‡ Data are expressed as mean±SE.

<sup>||</sup>  $P < 0.025$  compared to the control cultures.

prelabeled in one of two different ways. In the first experiment, cells were prelabeled for only 24 h, a time period which resulted in labeling the intracellular pool of both normal and DDEB cells to approximately the same extent. Under these circumstances, degradation of [<sup>35</sup>SO<sub>4</sub>]<sup>-</sup>glycosaminoglycans was the same in normal and albopapuloid DDEB cells (Fig. 4A). However, the existence of two metabolically distinct pools can be postulated, one in which glycosaminoglycans are degraded normally and the other which putatively contains glycosaminoglycans which are not readily degraded. Such a pool might take several days to accumulate as shown in Fig. 1. Thus, in a second set of experimental conditions, cells were prelabeled for 72 h, resulting in a two- to threefold increase in labeled material in the DDEB cells at the start of the chase period (Fig. 4B). Again, no significant differences in the rates of degradation were observed between the control and DDEB cultures.

The <sup>35</sup>SO<sub>4</sub><sup>-</sup>-labeled compounds lost from the cells during the chase period were quantitatively recovered in the medium. In the control line 22% of the labeled material was secreted into the medium in macromolec-

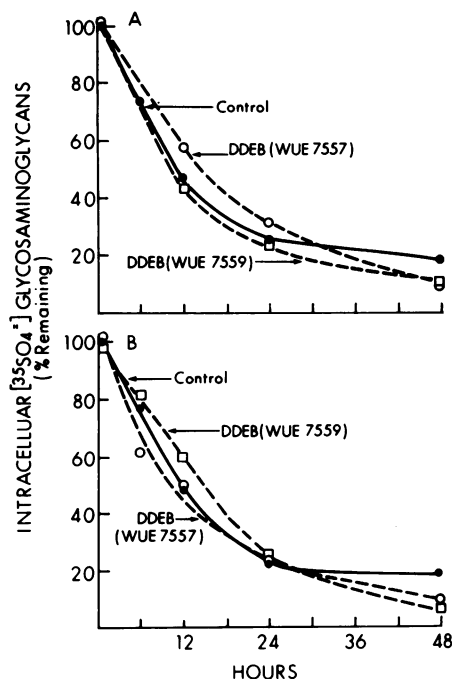


FIGURE 4 Loss of <sup>35</sup>SO<sub>4</sub><sup>-</sup>-labeled intracellular glycosaminoglycans. (A) Control and DDEB cell cultures were labeled with <sup>35</sup>SO<sub>4</sub><sup>-</sup> for 24 h after which the cells were passed and incubated in fresh medium at pH 6.8 for up to 48 h. Zero-time values were 11,279 cpm/mg protein for the control cells and 13,095 and 17,279 cpm/mg protein for WUE 7557 and WUE 7559, respectively. ●, control (CRL 1224); ○, albopapuloid DDEB (WUE 7557); □, albopapuloid DDEB (WUE 7559). (B) Control and DDEB cell cultures were labeled for 72 h and then processed as described in "A". Zero-time values were 10,109 cpm/mg protein for the control cells and 18,778 and 34,136 cpm/mg protein for WUE 7557 and WUE 7559, respectively. ●, Control (WUN 77201); ○, albopapuloid DDEB (WUE 7557); □, albopapuloid DDEB (WUE 7559). The data are expressed as the mean percent of radioactivity remaining.

ular form after a 24-h chase, whereas 78% was recovered as dialyzable <sup>35</sup>SO<sub>4</sub><sup>-</sup>. Similarly, in two albopapuloid DDEB cell lines 14 and 17% of the <sup>35</sup>SO<sub>4</sub><sup>-</sup>-labeled compounds was secreted as macromolecular material and 86 and 83%, respectively, existed as dialyzable <sup>35</sup>SO<sub>4</sub><sup>-</sup>-labeled compounds. These findings indicate that in the DDEB cultures the intracellular [<sup>35</sup>SO<sub>4</sub>]<sup>-</sup>glycosaminoglycans have been degraded to an extent equal to that of the control fibroblast cultures.

Experiments were also performed to determine if the accumulation defect in DDEB cells could be corrected in vitro as had been described with the single enzyme defect mucopolysaccharidoses (15). Albopapuloid DDEB and control fibroblasts were mixed in equal numbers and grown to confluence after which the intracellular accumulation of labeled glycosaminoglycans was determined (Fig. 5). The radioactivity incorporated

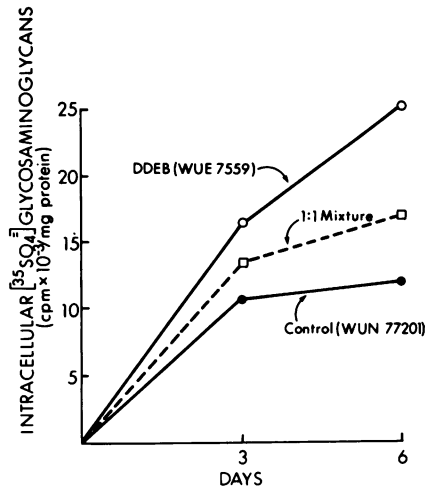


FIGURE 5 Coculture of normal and albopapuloid DDEB fibroblasts. Incorporation of  $^{35}\text{SO}_4^-$  into glycosaminoglycans was determined in control and DDEB fibroblasts as described in Methods and compared to the incorporation obtained with a 1:1 mixture of normal and DDEB cells in the same culture. ●, control (WUN 77201); ○, albopapuloid DDEB (WUE 7559); □, 1:1 coculture.

into a 1:1 coculture of DDEB and control cells was equal to the average of the two lines cultured separately, indicating no cross-correction of the defect.

The possibility that the increased accumulation of sulfated glycosaminoglycans was due to exaggerated sulfate transport was also assessed in the albopapuloid DDEB and control lines. After a brief exposure to  $\text{Na}_2^{35}\text{SO}_4$ , fibroblast cultures were immediately washed, placed on ice, and harvested by scraping. The lysed cells were then counted to determine the radioactivity which had been internalized. No differences in the rate of  $^{35}\text{SO}_4^-$  transport were observed with control lines having 56, 69, 55, and 71 intracellular cpm and a representative albopapuloid DDEB line having 54, 65, 46, and 51 intracellular cpm at 30, 60, 120, and 180 s, respectively.

To determine if the enhanced accumulation of  $^{35}\text{SO}_4^-$ -glycosaminoglycans was due to defective endocytosis from the medium, both control and DDEB cells were incubated with  $^{35}\text{SO}_4^-$ -labeled compounds prepared either from control or DDEB cells (12). The net (mean  $\pm$  SE) endocytosis by control cultures ( $n = 4$ ) of control-derived  $^{35}\text{SO}_4^-$ -labeled compounds was  $3,310 \pm 411$  cpm/7 h per mg protein ( $1.3 \pm 0.2\%$  of the added radioactivity) compared to  $3,307 \pm 688$  cpm/7 h per mg protein ( $1.0 \pm 0.2\%$  of the added radioactivity) in the DDEB cell lines ( $n = 5$ ;  $P = \text{NS}$ ). Similarly, using labeled material prepared in DDEB cultures, the net uptake by the control cells ( $n = 4$ ) was  $5,430 \pm 830$  cpm/7 h per mg protein ( $2.3 \pm 0.5\%$  of the added radioactivity) compared to an uptake in the DDEB cultures ( $n = 5$ ) of

$5,845 \pm 1,461$  cpm/7 h per mg protein ( $1.9 \pm 0.5\%$  of the added radioactivity) ( $P = \text{NS}$ ). Thus, differences in the uptake between control and DDEB cultures did not account for greater accumulation of glycosaminoglycans.

The molecular weight characteristics of intra- and extracellular  $^{35}\text{SO}_4^-$ -labeled compounds were determined by gel filtration (Fig. 6). The elution patterns of extracellular material from a control (Fig. 6A) and two different albopapuloid DDEB cell lines (Fig. 6B, C) were essentially identical. The two DDEB cell lines displayed a higher proportion of intracellular material eluting between the exclusion volume of the column

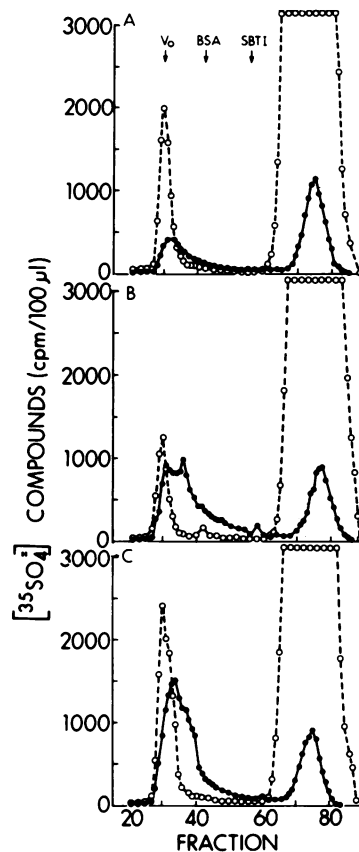


FIGURE 6 Gel filtration of labeled glycosaminoglycans from control and albopapuloid DDEB cultures. Cultures were labeled for 72 h before harvesting the cells and medium. Aliquots of culture medium or cell sap were placed on a  $1.6 \times 35$ -cm column of Sephadex G-200 equilibrated with  $0.02 \text{ M } (\text{NH}_4)_2\text{SO}_4$  and eluted at a rate of 10 ml/h. ○, extracellular  $^{35}\text{SO}_4^-$ -labeled compounds; ●, intracellular  $^{35}\text{SO}_4^-$ -labeled compounds. (A) Control (WUN 77201): 1 ml of cell lysate or 1 ml of medium was applied to the column in two different gel filtrations; (B) WUE 7557: 1 ml of cell lysate or 0.5 ml of medium was applied to the column in two different filtrations; (C) WUE 7559: 1 ml of cell lysate or 0.5 ml of medium was applied to the column in two different filtrations. BSA and SBTI refer to elution positions of bovine serum albumin and soybean trypsin inhibitors, respectively.

and the elution position of bovine serum albumin (i.e., fractions 32–45), although the increase in intracellular material was not confined to this region alone.

If increased accumulation of glycosaminoglycans were, indeed, a genetic characteristic of alpopapuloid DDEB fibroblasts, it might be a useful *in vitro* marker for aiding in the classification of EB patients. This possibility was explored by comparing the intracellular [ $^{35}\text{SO}_4^-$ ]glycosaminoglycan content of alpopapuloid DDEB cells with that of cells from other forms of EB (Fig. 7). After a 48-h labeling period, the seven alpopapuloid DDEB fibroblast lines had greater than a two-fold increase in labeled material compared to control cultures ( $P < 0.001$ ). In contrast, cell lines initiated from patients with dominant EB simplex and recessive dystrophic EB had values which were 95 and 98% of the control cultures, respectively. Furthermore, it is noteworthy that cells from three patients from two kindreds with another form of DDEB, not of the alpopapuloid variety, did not manifest increased accumulation of labeled glycosaminoglycans.

## DISCUSSION

The present study demonstrates that fibroblasts taken from patients with the alpopapuloid variety of DDEB manifest a defect in glycosaminoglycan metabolism *in vitro*. This aberration is characterized by an increased accumulation of sulfated glycosaminoglycans both as intra- and extracellular material. The elevation could result either from increased synthesis or decreased deg-

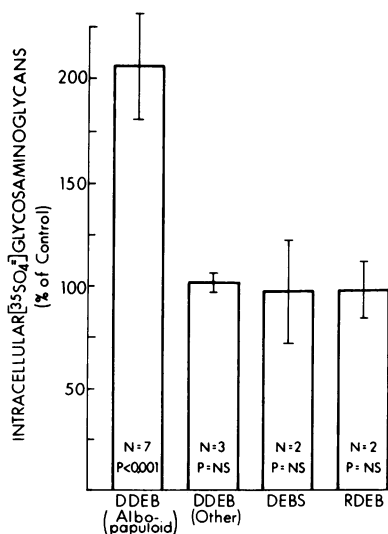


FIGURE 7 Intracellular accumulation of labeled glycosaminoglycans in various types of EB. Incorporation of  $^{35}\text{SO}_4^-$  into glycosaminoglycans was determined after a 48-h labeling period. Accumulation was determined as counts per minute per milligram protein in eight different experiments. All of the EB fibroblast lines are those noted in Table I.

radation. However, several pieces of evidence suggest the excessive accumulation of [ $^{35}\text{SO}_4^-$ ]glycosaminoglycans is due to increased synthesis. (a) The initial rate of accumulation of intracellular labeled material was greater in DDEB cells than either in control or in other EB fibroblasts at a time when measurement of the rate of accumulation is not substantially complicated by breakdown (Fig. 2). (b) Secretion of [ $^{35}\text{SO}_4^-$ ]glycosaminoglycans into the medium occurred at a significantly greater rate than in control lines (Fig. 3, Table II). (c) The turnover of labeled glycosaminoglycans in the intracellular pool of the DDEB cells was equal to that of control cells irrespective of whether the DDEB cultures were prelabeled to the same extent (Fig. 4A) or were allowed to accumulate two- to threefold greater  $^{35}\text{SO}_4^-$ , thus, enlarging the labeled degradative pool (Fig. 4B). This finding suggests that the DDEB cells do not contain two metabolically distinct pools for degradation of glycosaminoglycans. (d) DDEB and control cells demonstrated an identical rate of uptake of  $^{35}\text{SO}_4^-$ -labeled compounds, suggesting that defective endocytosis is not the underlying mechanism for excessive extracellular accumulation. (e) Unlike the defect in various mucopolysaccharidoses, where cocultivation of cells deficient for different single enzymes leads to cross-correction (15), no *in vitro* cross-correction of the DDEB defect was observed (Fig. 5). However, this type of experiment does not exclude a degradative defect involving many enzymes, such as mucopolipidosis II, or one in which there is poor transfer of enzyme between cells.

The increased accumulation of glycosaminoglycans appears to be a specific manifestation of the mutant alpopapuloid DDEB cells. Fibroblasts from other, genetically distinct forms of EB, such as recessive dystrophic EB and dominant EB simplex, failed to show any abnormality in glycosaminoglycan accumulation (Figs. 2, 3, and 7). More importantly, even the cells from patients with a different subvariety of DDEB did not manifest the abnormality, indicating that the characteristic is genetically unique for alpopapuloid DDEB. It is important to stress that the present studies were performed using cells taken only from normal-appearing skin. In no case were the biopsies taken from blistered areas or from the alpopapuloid lesions in the patients with DDEB. Thus, the aberration in glycosaminoglycan metabolism is not due to inadvertent cellular selection and is almost assuredly a genetically determined characteristic.

Since the phenotypic characteristics of the dominant and recessive forms of dystrophic EB may be quite similar early in life (1, 2), a specific biochemical marker could be of important prognostic value in the sporadic patient with EB. The apparent specificity of this *in vitro* trait suggests that it might be a useful diagnostic aid in dominant dystrophic EB. In this regard, we have ex-



aminated fibroblasts from a 1-yr-old sporadic patient whose disease clinically suggests that she may represent a new mutant for DDEB, although she is still too young to have developed allopapuloid lesions. After 48 h in culture, the mean intracellular labeled glycosaminoglycan content in five experiments was  $200.3 \pm 29.0\%$  of control (mean  $\pm$  SE;  $P < 0.001$ ), indicating that the patient may, indeed, have DDEB of the allopapuloid variety.

In the broader sense, the present studies emphasize the biochemical heterogeneity underlying the various forms of EB. Although an etiology has not been defined for any of the types of EB, fibroblast cultures have been useful in elucidating certain, apparently specific, genetic characteristics. In the case of recessive dystrophic EB, in at least three patients, cultured fibroblasts have been shown to synthesize increased amounts of an altered form of collagenase (16), and the data indicate that this finding is unique for recessive dystrophic EB (17). Similarly, in the present study the increased synthesis of glycosaminoglycans was found only in cells from patients with allopapuloid DDEB. It is, thus, conceivable that these biochemical markers might now be utilized for further genetic studies and for antenatal diagnosis in kindreds at risk.

The relationship of the abnormality in glycosaminoglycan metabolism to the blistering phenomenon is, as yet, unknown. As suggested by McKusick (18), however, in dominant diseases (such as DDEB), an alteration in the synthesis of a critical structural macromolecule might well be expected to be expressed as an abnormal phenotype. In this regard, there is considerable evidence to indicate that glycosaminoglycans interact with collagen to influence both the rate and quality of collagen fibril formation (19). Indeed, whereas some mucopolysaccharides promote fibril formation (20, 21), others hinder it (22). It may be that accumulation of glycosaminoglycans, which interact with collagen to lead to aberrant fibril formation, impairs the structural integrity of the skin and causes the posttraumatic blisters and erosions characteristic of allopapuloid DDEB.

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