Expression of Basement Membrane Gene in Cultured Skin Fibroblasts from Patients with Diabetes Mellitus

Byung Chun Kim, M.D., Kyu Suk Lee, M.D.

Department of dermatology, Keimyung University School of Medicine, Taegu, Korea

Background **: Bullous eruption of diabetes(BD) is a rare cutaneous sign of diabetes mellitus(DM). The mechanism for the development of these lesions is unknown, although speculation has ranged from trauma to vascular insufficiency.**

Objective **: Our purpose is to evaluate the difference of basement membrane gene expression in cultured skin fibroblasts between patients with diabetes and normal controls.**

Methods **: Total RNA was extracted from cultured skin fibroblasts of DM and normal, and then Northern blot and slot-blot hybridizations were done.**

Results **: The mRNA levels of α(I) procollagen, α(IV) procollagen, fibronectin, and laminin B1 were not altered significantly in the DM.**

Conclusion **: Our results suggest that BD has no relevance to the alteration of basement membrane components. Further studies are needed to clarify the underlying pathogenic mechanism of BD. (Ann Dermatol 8:(1) 1~5, 1996).**

Key Words : Basement membrane gene, Diabetes Mellitus

Cutaneous manifestations are frequently seen in 30% of diabetic patients. The occurrence of recurrent bullous lesions, especially on the extremities in diabetic patients, is a well established but rare complication[1,2]. Many investigators have reported that a high serum glucose level induces an alteration of extracellular matrix components[3-5] and gene expression in renal glomerular cells[6-11], but the cause of bullous diabeticorum(BD) is still unknown. Although a lower threshold to suction blistering may suggest a specific weakness in diabetic skin and increased susceptibility to trauma[12], little is known about basement membrane(BM) gene expression in diabetic skin. Therefore, we studied BM gene expression in cultured human skin fibroblasts from diabetic patients using Northern transfer and slot-blot hybridization analyses.

Received June 2, 1995.
Accepted for publication September 21, 1995.
Reprint request to : Byung Chun Kim, M.D., Department of dermatology, Keimyung University School of Medicine, Taegu, Korea

MATERIALS AND METHODS

Fibroblast culture

Skin samples from 5 complicated diabetes mellitus(DM) patients and 5 normal controls(N) were obtained by 4mm punch biopsies on the medial aspect of the left upper arm. Primary culture of the dermal fibroblasts were established by routine methods, and subcultivated on plastic culture dishes in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin(100 U/ml), streptomycin(100 μ g/ml), and amphotericin B(1 μg/ml). The cells were maintained in a humidified 5% CO^2 - 95% air incubator at 37℃. Analyses of fibroblast cultures were carried out at 3 - 6 passages of subcultivation. The cases are summarized in Table 1.

cDNA preparation

The following human-sequence-specific cDNA were utilized in our study: for α(I) collagen;a 1.8 kb α(I) collagen cDNA, for α(IV) collagen;a 2.6 kb α (IV) collagen, for fibronectin(FN);a 1.3 kb fibronectin cDNA, for laminin(Lam) B1;a 2.4 kb

Annals of Dermatology
Vol. 8, No. 1, January 1996

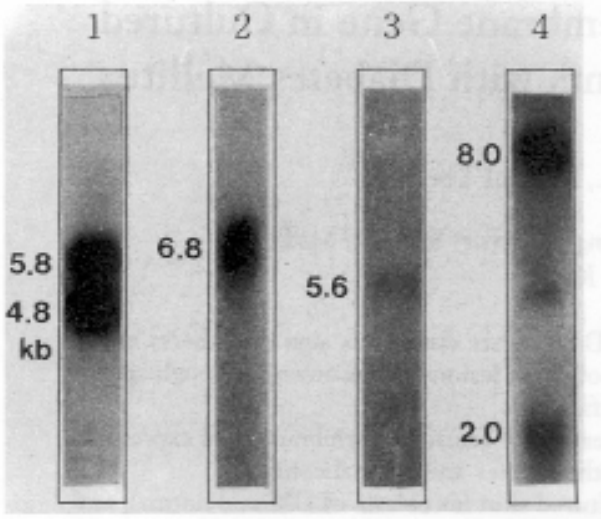

Fig. 1. Northern transfer analysis of α1(I) procollagen (lane1), α 1 (IV) procollagen (lane2), laminin B1 (lane3), and fibronectin and β-actin (lane4) mRNA transcripts from normal skin fibroblast cultures. In lane 4, the faint 5.6 Kb band for laminin B1 is due to incomplete washing after rehybridization.

laminin B1 cDNA, for β-actin;a 1.7 kb β-actin cDNA. The cDNAs labeled with ^{32}P-dCTP(NEG 036H, New England Nuclear, Boston, USA) by nick translation[13] to a specific activity of approximately 1 × 10^8 cpm/μg.

RNA isolation

Total RNA was isolated by the methods of Chomzynski and Sacchi[14] from cultured diabetic and normal skin fibroblasts. Total RNA lysed directly by the addition of guanidium-thiocyanate buffer(4M guanidium thiocyanate, 5mM Na-citrate, PH 7.0, 0.5% sarcosyl, 0.1M-2-mercatoethanol, 0.33% antifoam A emulsion), followed by phenol extraction and ethanol precipitation. RNA pellet was suspended in diethylpyrocarbonate treated water and concentration of RNA was determined by measuring absorbance at 260nm and the purity of nucleic acid preparation was assessed by the 260/280nm ratio.

Northern blot and slot-blot hybridizations

The RNA was subjected to both Northern blot and slot-blot hybridization analyses. Total RNA was fractionated by 1% agarose gel electrophoresis(50 volt, 5 hours) after denaturating the samples with formaldehyde and formamide[15]. The

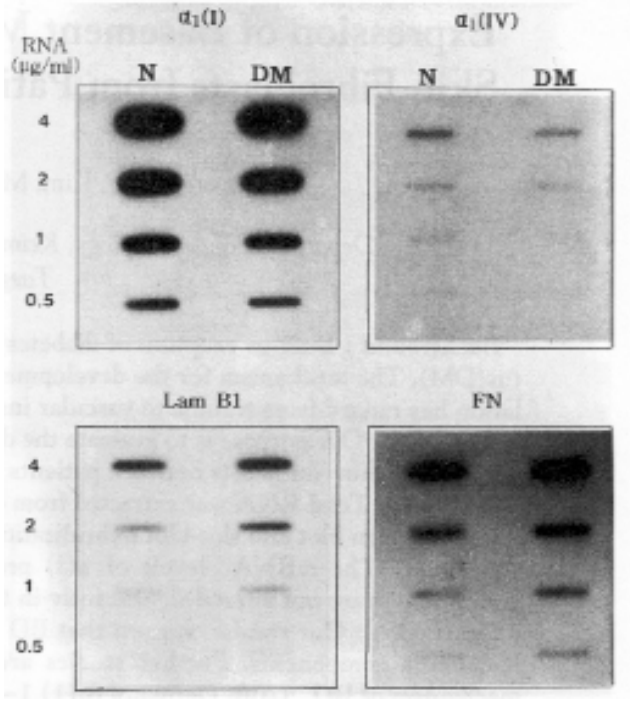

Fig. 2. Slot-blot hybridization of RNA isolated from normal(N)or diabetic(DM) skin fibroblst cultures. Total RNA was isolated, and different amounts, 4.0, 2.0, 1.0, and 0.5ß, were dotted on to nitrocellulose filters.

RNA transcripts obtained were transferred to the nitrocellulose filter(Trans-Blot, BioRad, Richmond, USA) in 20 × SSC overnight at 4℃[16]. The samples for the slot-blot analyses were denatured with formaldehyde, and 4 different dilutions from 4.0, 2.0, 1.0, 0.5 μg of total RNA were dotted onto a nitrocellulose filter using a slot-blot vacuum manifold(Minifold 2, Schleicher & Schuell, Dassel, Germany). Then each filter was prehybridized for 12-18 hours at 42℃ with a prehybridization mixture(50% formamide, 0.1% SDS, 3 × SSC, 1 × Denhart'solution, 50 μ g/ml ss-DNA) and hybridized with ^{32}P-labeled cDNA by nick translation at 42℃ for 24 - 36 hours.

Following hybridization, washing and autoradiography was performed. The corresponding steady-state levels of mRNA were quantitated with a laser densitometer(LKB Instrument, Inc., Bromma, Sweden).

Statistical analysis

Differences between DM and N skin fibroblasts were analyzed using Students t test for paired variables.

Table 1. Clinical features of diabetic patients

No.	Age/Sex	Duration	Type	Complication	Glucose(mg/dl)
1.	45/M	3 mo.	NIDDM	PN	413
2.	63/M	3 yrs	NIDDM	FU, NPH	193
3.	56/M	6.5 yrs	NIDDM	FU, PN	223
4.	33/M	10 yrs	IDDM	FU, RTP, PN	327
5.	59/F	1 yrs	NIDDM	CL, PN	444

PN : Peripheral Neuropathy, FU : Foot Ulcer, NPH : Nephropathy
RTP : Retinopathy, CL : Cellulitis

Table 2. Quantitation of α1(I), α1(IV) collagen, FN and Lam B1 mRNA levels in cultured skin fibroblasts from patients with diabetes and normal controls

	α1(IV)	FN	Lam B1	α1(I)
N (n=5)	209 ±5.0	375.9 ± 4.73	273.0 ±8.72	1206.3 ± 66.3
DM(n=5)	200 ±13.2*	387.3 ±2.52*	290.3 ±8.08*	1296.7 ± 90.74*

The values are mean ±SD and expressed as densitometric absorbance unit which are the percentage of the values of β-actin
* not significantly different from normal controls ($p > 0.05$)

RESULTS

In Northern blot analysis, ^{32}P labeled α(I) procollagen, α (IV) procollagen, laminin B1, fibronectin and β-actin cDNA probes hybridized with mRNA specially. α(I) procollagen revealed 5.8 & 4.8 kb polymorphic transcripts and α(IV) procollagen showed 6.8 kb monomorphic transcript. The molecular sizes of laminin B1, fibronectin and β-actin mRNA revealed 5.6 kb, 8.0 kb, and 2.0 kb, respectively, in N skin fibroblasts(Fig. 1).There was no change of sizes of mRNAs between DM and N skin fibroblasts(N:Fig. 1, DM:data not shown).

Type I, type IV, FN and Lam B1 mRNA levels were measured by quantitative slot-blot hybridization. For each sample, the density of the band representing α(I) procollagen, α(IV) procollagen, FN, laminin B1 mRNAs was divided by the density of that for β-actin mRNA to normalized for inter-sample variations in the amounts of total mRNA loaded. Little, if any, change was noted in steady-state mRNA levels of α(I) procollagen, α(IV) procollagen, FN, and Lam B1 with DM and N skin fibroblasts by Students t test(Fig. 2, and Table 2).

DISCUSSION

Cutaneous manifestations of DM affect approximately 30% of diabetic patients, such cutaneous signs of DM include necrobiosis, pruritus, pyoderma and bullous eruption[17]. In fact, the actual prevalence of skin manifestations probably approaches 100%, especially if metabolic effects on the microcirculation and changes in skin collagen are involved. BD have rarely been reported in diabetic patients, the bullae, usually multiple, appear on the extremities overnight without any preceding trauma[1,2]. The cause of BD is still unknown. Many authors accept a relationship with vascular or neurologic disturbances[18-20]. Most patients have long standing diabetes and vascular and/or neurologic complications occur. However such a relationship is not present in all cases of BD. All our 5 cases have complications such as peripheral neuropathy, nephropathy, and retinopathy, and the disease is varying in duration from 3 months to 10 years. Other authors[17] speculate that nephropathy might be more significant, with a resulting imbalance in Ca^{++} and Mg^{++} and subsequent weakness in skin structure, also a reduced threshold to suction induced blister formation in diabetic patients has

been observed[12], electron microscopic examination showing the separation at the level of the lamina lucida. The same findings were observed in earlier studies[17,21]. This lowered threshold to suction blistering may suggest a specific weakness in diabetic skin and an increased susceptibility to trauma, the main location of BD on the distal extremities indicates that minor frictional or physical trauma might play at least a partial role. However, many authors[17-20] have stated that there was no previous trauma history and bullae usually developed overnight, thus trauma is unlikely to be the only causative factor.

It is now well established that diabetic glomerulopathy is characterized by BM thickening and mesangial expansion[22], furthermore it is evident that an imbalance occurs in the components of extracellular matrices manifested by an increase in type VI,IV collagen[23] and reduction in the content of heparin sulfate and laminin[11]. Also in other studies, the mRNA levels for α(IV), α(I), α(III) collagen chains, FN, and Lam B1, B2 were increased or unchanged[6-11], and there is a known hypothesis that increased nonenzymatic glycosylation of Lam and type IV collagen can alter the macromolecular assembly of BM or other extracellular matrices[24]. Therefore an alteration in the components of epidermal BM and BM gene expression may be postulated as a possible cause of BD, but little is known about BM and BM gene expression in diabetic skin except for some earlier works[25,26]. In Northern blot analysis, α(I) procollagen, α(IV) procollagen, Lam B1, and FN revealed no change in size, which indicates no alteration in its quality. Densitometric analysis of the autoradiograms from slot-blot hybridizations showed that there was little, if any, change in steady-state mRNA levels of α(I) procollagen, α(IV) procollagen, FN, and Lam B1 with DM and N skin fibroblasts, after the mRNA levels were corrected for the β-actin mRNA abundance in the same RNA preparation.

In conclusion, BM gene expression in complicated DM does not differ from normal controls, so our results suggest that BD has no relevance to the alteration in BM gene expression. Further work in the structure and function of BM, as well as the molecular defect of BM are needed to clarify the underlying mechanism of BD, as increased nonenzymatic glycosylation of BM components such as laminin and type IV collagen and changes in their molecular association with heparan sulfate proteoglycan may be the phenomena that link hyperglycemia to changes in the structure and function of BM in diabetes.

REFERENCES

1. Gilgor RS, Lazarus GS : Skin manifestations of diabetes mellitus. In Rifkin H, Raskin P (eds) : Diabetes mellitus. R J Brady Co, Maryland, 1981, pp. 313-321.
2. Perez MI, Kohn SR : Cutaneous manifestations of diabetes mellitus. J Am Acad Dermatol 30: 519-531, 1994.
3. Rohrbach DH, Wagner CW, Star VL, Martin GR, Brown KS, Yoon JW : Reduced synthesis of basement membrane heparan sulfate proteoglycan in streptozocin induced diabetic mice. J Biol Chem 258:11672-11677, 1983.
4. Sternberg M, Cohen-Forterre L, Peyroux J : Connective tissue in diabetes mellitus: Biochemical alterations of the intercellula matrix with special reference to proteoglycan, collagens and basement membrane. Diabete Metab 11: 27-50, 1985.
5. Shimomura H, Spiro RG : Studies on macromolecular components of human gromerular basement membrane and alteration in diabetes : Decreased levels of heparan sulfate proteoglycan and laminin. Diabetes 36:374-381, 1987.
6. Ledbetter S, Copeland EJ, Noonan D, Vogeli G, Hassell JR : Altered steady-state mRNA levels of basement membrane proteins in diabetic mouse kidneys and thromboxane synthetase inhibition. Diabetes 39:196-203, 1990.
7. Puolsom R, Kurkinen M, Prockop DJ, Boot-Handford RP : Increased steady-state levels of laminin B1 mRNA in kidneys of long-term streptozocin-diabetic rats : No effect of an aldolase reductase inhibtor. J Biol Chem 263:10072-10076, 1988.
8. Zyradeh FN, Snipes ER, Watanabe M, Alvarez RZ, Goldfarb S, Haverty TP : High glucose induces cell hypertrophy and stimulates collagen gene transcription proximal tubule. Am J Physiol 259:F704-714,1990.
9. Zyradeh FN, Simmons DR, Snipes ER, Goldfarb S : Effects of myoinositol on cell proliferation and collagen transcription and secretion in proximal tubule cells cultured in elevated glucose. J Am Soc Nephrol 1:1220-1229,1991.
10. Cagliero E, Maiello M, Boerl D, Roy S, Lorenzi M :

Increased expression of basement membrane components in human endothelial cells cultured in high glucose. J Ciln Invest 82:735-738, 1988.
11. Ayo SH, Radnik RA, Glass WF II, Garonl JA, Rampt ER, Appling DR, Kreisberg JI : Inceased extracellular matrix synthesis and mRNA in mesangial cells grown in high-glucose medium. Am J Physiol 260:185-191, 1991.
12. Bernstein JE, Levine LE, Medenica MM et al : Reduced threshold suction induced blister formation in insulin-epidermolysis bullosa without immunoreactants. J Am Acad Dermatol 8:790-791, 1983.
13. Pigby DNJ, Dieckmann M, Rhodes L : Labeling deoxyribonucleicacid to high specipic in vitro by nick-translation with DNA polymerase J Mol Biol 113:237-251, 1977.
14. Chomczynski P, Sacchi N : Single step method of mRNA isolation by acid guanidium, thiocyanate-phenol-chlorform extraction. Anal Bio chem 162:156-159, 1987.
15. Whal GM, Stam M, Starck GR :Efficient transfer of large DNA fragments from agarose gel to diabenzyl-loxymethyl paper and rapid hybridization by suing dextra sulfate. Proc Natl Sci USA 76:3683-3687, 1979.
16. Thomas PS : Hybridization of denatured RNA and small DNA fragments transfered to nitrocellulose. Proc Natl Sci USA 77:5201-5205,1980.
17. Bernstein JE, Medenica M, Soltani K, Griem SF : Bullous eruption of diabetes mellitus. Arch Dermatol 115:324-325, 1979.
18. Cantwell AR, Martz W : Idiopathic bullae in diabetes : Bullous diabeticorum. Arch Dermatol 96:42-44, 1967.
19. Bear CL, Wall LH : Idiopathic bullae in diabetes. Australas J Dermatol 10:33-37, 1969. (cited from reference 17.)
20. Kurwa A, Roberts P, Whitehead R : Concurrence of bullous and atrophic skin lesions in diabetes mellitus. Arch Dermatol 103:670-675, 1971.
21. Toonstra J : Bullous diabeticorum. J Am Acad Dermatol 13:799-805, 1985.
22. Mauer SM, Steffes MW, Brown DM :The kidney in diabetes. Am J Med 70:603-612, 1981.
23. Mohan PS, Carter WG, Spiro RG : Occurrence of type IV collagen in extracellular matrix of renal glomeruli and its increase in diabetes. Diabetes 39:31-37, 1990.
24. Joseph FT, Lorrel AR, Leo TF : Molecular mechanisms in basement membrane complications of diabetes : Alteration in heparin, laminin, and type IV collagen association. Diabetes 37:532-539, 1988.
25. Rowe DW, Starman BJ, Fujimoto WY, Williams RH : Abnormalities in prolifreration and protein synthesis in skin fibroblast cultures from pattients with diabetes mellitus. Diabetes 26:248-290, 1977.
26. Silbert CK, Kleimann HK : Studies of cultured human fibroblasts in diabetes mellitus : Changes in heparan sulfate. Diabetes 28:61-64, 1979