Expression of the Extracellular Matrix Gene in Response to Insulin and Insulin-like Growth Factor in Cultured Skin Fibroblasts

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Background: **Diabetes mellitus is a heterogeneous group of disorders characterized by high serum glucose levels and by disturbances of carbohydrate and lipid metabolism. There are many cutaneous signs of this common endocrinopathy, such as nercobiosis lipoidica diabeticorum, diabetic bullosis, shin spot, diabetic pruritus, etc.**

Objective: **In this study, we investigated whether extracellular matrix gene expression in cultured skin fibroblast is influenced by insulin and Insulin-like growth factor-I(IGF-I).**

Method: **Total RNA was isolated from insulin or IGF-I treated human skin fibroblasts. The Northern blot and slot-blot hybridization were then conducted.**

Results: **The mRNA levels of pro α1(I) collagen, pro α1(III) collagen, fibronectin in insulin and IGF-I treated normal skin fibroblasts increased compared with untreated normal skin fibroblasts.**

Conclusion: **Our results show that insulin and IGF-I stimulate collagen formation in normal skin fibroblast at physiological concentrations. Therefore, these demonstrate that insulin can modulate the expression of extracellular matrix gene.(Ann Dermatol 7:(4)303~307, 1995)**

Key Words : Diabetes mellitus, Insulin, Insulin-like growth factor, Extracellular matrix gene

Diabetes mellitus is a heterogeneous group of disorders characterized by high serum glucose levels and by disturbances of carbohydrate and lipid metabolism. There are many cutaneous signs of this common endocrinopathy such as nercobiosis lipoidica diabeticorum, diabetic bullosis, shin spot, diabetic pruritus, etc[1].

Insulin and insulin-like growth factor(IGF) contribute to the repair of the extracellular matrix after injury to endothelial or epithelial surfaces[2-6]. IGF-I is also a paracrine factor which is synthesized by fibroblasts and smooth muscle cells[6,7]. These molecules influence inflammatory reactions by stimulating the growth of fibroblasts and the production of matrix molecules[2,6,8].

In diabetic animals not treated with insulin, the collagen content of the skin and vascular tissue is decreased[9], suggesting that insulin may contribute to the normal regulation of the extracellular matrix.

In this study, we investigated whether extracellular matrix gene expression in cultured skin fibroblast is influenced by insulin and IGF-I, using the Northern blot and slot-blot hybridizations.

MATERIALS AND METHODS

Fibroblast Culture

Cells were grown in dulbecco's modified Eagle's medium(DMEM) with 10% fetal bovine serum, penicillin(100 U/ml), streptomycin(100μg/ml), and amphotericin B(1μg/ml). The cells were maintained in a humidified 5% CO_2-95% air incubator at 37°C. Trypan blue test was used for checking cell viability.

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Treatment of insulin, IGF-I

Confluent fibroblasts in 100mm diameter petridishes were treated with insulin(24μU/ml, Sigma Co. ST. Louis, USA) or IGF-I(100ng/ml, Amgen, Thousand Oaks, CA, USA) for 48 hours in the absence of fetal bovine serum.

cDNA Preparation

The following human-sequence-specific cDNAs were utilized for this study: for α1(I) collagen: a 1.8kb α1(I) collagen cDNA; for α1(III) collagen: a 1.32-kb α1(III) collagen cDNA; for Fibronectin: a 1.3-kb Fibronectin cDNA; for GAPDH: a 1.2-kb GAPDH cDNA, The cDNAs were labeled with ^{32}P-dCTP(NEG 0.36H, New England Nuclear, Boston, USA) by nick translation[10] to a specific activity of approximately 1×10^8cpm/μg.

cDNA	Vector	Enzyme	Insert size(Kb)	mRNA size(kb)
α1(I) collagen	pBR322	Eco RI	1.8	5.8, 4.8
α1(III) collagen	pBR322	Eco RI	1.32	5.5, 4.8
Fibronectin	pBR322	Eco RI	1.3	8.0
GAPDH	pBR322	Pst I, Hind III	1.2	1.4

RNA Isolation

Total RNA was isolated using the methods of Chomzynski and Sacchi[11] from cultured 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-mercaptoethanol, 0.33% antifoam A emulsion), followed by phenol extraction and ethanol precipitation. RNA pellet was suspended in diethylpyrocarbonate treated water and the 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 and Slot-Blot Hybridiazation

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[12]. RNA transcripts obtained were transferred to the nitrocellulose fiter(Trans-Blot, BioRad, Richmond, USA) in 20 X SSC overnight at 4°C[13]. The samples for the slot-blot analyses were denatured with formaldehyde, and 4 different dilutions from 4.0, 2.0, 1.0, 0.5μg of total RNA were dotted onto a nitrocellulose filter using a slot-blot vaccum manifold(Minifold II, Schleicher & Schuell, Dassel, Germany). Then each filters was prehybridized for 12-18 hours at 42°C with prehybridization mixture(50% formamide, 0.1% SDS, 3 X SSC, 1 X Denhart's solution, 50μg/ml ss-DNA) and hybridized with ^{32}P-labeled cDNA by nick translation at 42°C for 24 to 36 hours. Followig hybridization, washing and autoradiography were performed.

Statistics

Statistical examination of the data was performed using Student's *t* test for means of equal and unequal sizes[14]. $P<0.05$ was considered significant.

RESULTS

In the Northern blot anlysis, ^{32}P labeled pro α 1(I) collagen, pro α 1(III) collagen, fibronectin and GAPDH cDNA probes hybridized with each mRNAs specifically. pro α1(I) collagen and pro α 1(III) collagen revealed two mRNA transcripts, which size were 5.8-kb, 4.8-kb and 5.5-kb, 4.8-kb, and fibronectin, GAPDH revealed one transcript, each size was 8.0-kb and 1.4-kb in insulin or IGF-I treated normal skin fibroblasts(Fig. 1,2). There was no change in it's size, which indicates there was no alteration of it's quality.

Type I, type III and fibronectin mRNA levels were measured by quantitative slot-blot hybridization(Fig. 3). For each sample, the density of the band representing pro α1(I), pro α1(III) collagen and fibronectin mRNAs was divided by the density of that for GAPDH mRNA to normalized for intersample variations in the amounts of total mRNA loaded. Steady-state levels of pro α1(I) collagen,

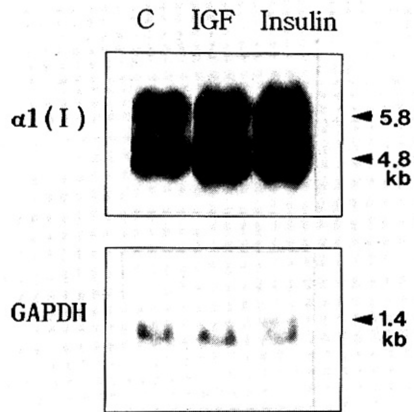

Fig. 1. Northern blot analysis of α1(I) collagen and GAPDH mRNA transcripts from control(C), insulin-like growth factor(IGF) or insulin treated fibroblasts in cultures.

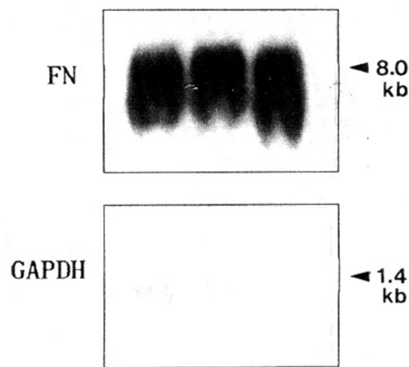

Fig. 2. Northern blot analysis of fibronectin(FN) and GAPDH mRNA transcripts from control(C), insulin-like growth factor(IGF) or insulin treated fibroblasts in cultures.

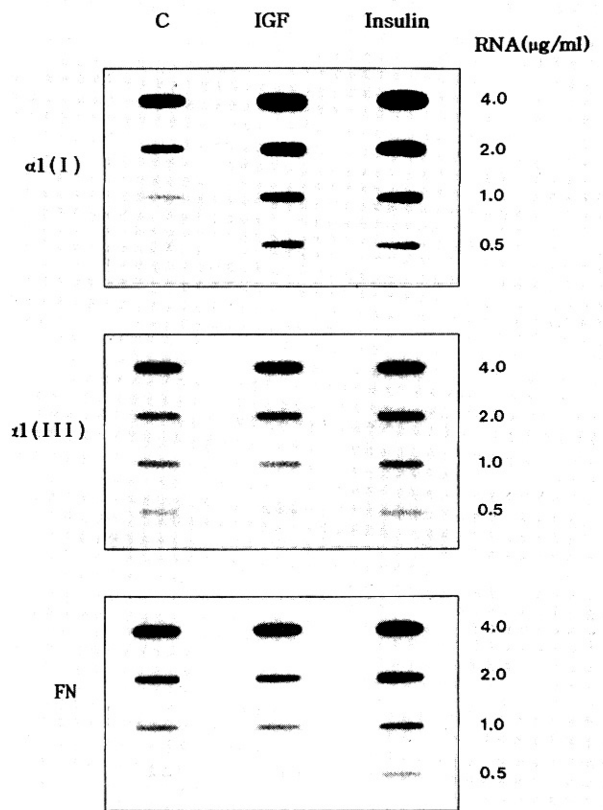

Fig. 3. Slot-blot hybridization of α1(I), α1(III) collagen and fibronectin(FN) RNA isolated from control(C), insulin-like growth factor(IGF) or insulin treated fibroblasts in cultures. Total RNA was isolated, and different amounts, 4.0, 2.0, 1.0, 0.5μg, were dotted on the nitrocellulose filters.

pro α1(III) collagen and fibronectin mRNAs were significantly increased in insulin or IGF-I treated normal skin fibroblasts(Table 1). In insulin treated normal skin fibroblasts, 2.19 ± 0.20, 1.50 ± 0.12, 1.47 ± 0.10-fold increased respectively(all, n=3, $p<0.01$), and in IGF-I treated normal skin fibroblasts, 1.39 ± 0.82(n=3, $p<0.01$), 1.22±0.06(n=3, $p<0.01$), 1.14 ± 0.09-fold(n=3, $p<0.05$) increased, respectively, as compaired with the control groups that were not treated(Fig. 4).

DISCUSSION

Diabetes mellitus is a heterogeneous group of disorders characterized by high serum glucose levels and by disturbances of carbohydrate and lipid metabolism. There are many cutaneous signs of this common endocrinopathy[1]. At least 30% of persons with diabetes have some type of cutaneous invovement during the course of their chronic disease[15].

However the pathogenesis of most of these cutaneous lesions is still not clearly understood, Necrobiosis lipoidica diabeticorum(NLD) that is

Table 1. Quantitation of α 1(I), α 1(III) collagen, fibnronectin(FN) mRNA levels in control, insulin-like growth factor(IGF) or insulin treated fibroblasts in cultures

	α1(I)/GAPDH	α1(III)/GAPDH	FN/GAPDH
Control(n=3)	180.7±11.0	100.4±4.0	150.4±8.1
IGF(n=3)	250.6±8.9*	122.8±9.7*	170.4±8.0**
Insulin(n=3)	400.0±13.9*	150.4±9.0*	219.6±8.359*

The values are mean ± SD and expressed as densitometric absorbance unit which are the percentage of the values of GAPDH.
* significantly different from control(p<0.01)
**significantly different from control(P<0.05)

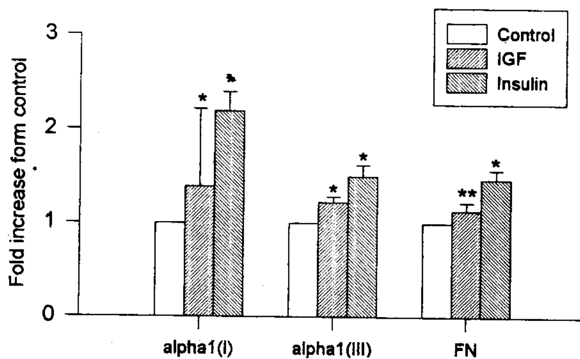

Fig. 4. Steady state levels α1(I), α1(III) collagen and fibronectin(FN) mRNA in control(C), insulin-like growth factor(IGF) treated or insulin treated fibroblasts in cultures. α1(I), α1(III) collagen and fibronectin(FN) /GAPDH mRNA ratio was compared with ratio from mRNA obtained from control cells not treated. The values are mean±SM. *p<0.01, **p<0.05, compared with control.

one of the diabetic skin diseases, is characterized by sharply defined erythematous plaques with a depressed, waxy, yellow-brown, atropic, telangiectatic center and 85 to 90% occur on the lower extremities, especially in the pretibial areas[16,17]. The pathogenesis of NLD is unknown, it has been reported that an immunologic reaction to an unknown stimulus may trigger events leading to NLD; release of cytokines from inflammatory cells or from tissue factors by injured endothelial cells may lead to degeneration of the connective tissue matrix and to decreased synthesis of collagen by afffected fibroblasts[1]. Expecially, Oikarinen A et al[18] has suggested that the reduced synthesis of collagen in skin fibroblasts of NLD patients was found to be due to a decreased amount of collagen mRNA, indicating that collagen synthesis was afffected at the pretranslational level.

Serum and cell derived effector molecules contribute to the response of extracellular matrix after injury to endothelial or epithelial surfaces[2]. Insulin and IGF are two such substances which circulate in blood[3-6]. The physiologic serum concentration of insulin is 7-24μU/ml on overnight fasting state and IGF is present in human serum in concenturations of up to 1μg/ml[6,19]. These molecules influence inflammatory reactions by stimulating the growth of fibroblasts and the production of matrix molecules[2,6,8]. Studies of the connective tissue matrix in diabetic animals suggest that insulin is important in maintaining the normal turn-over of extracellular matrix. The collagen content of skin and vascular tissue was decreased in diabetic animals[9,17] and treatment of diabetic animals with insulin resulted in a return of collagen content to normal levels[9]. Also, Ronald H et al[20] found that insulin and IGF-I stimulated the accumulation of collagen in human lung fibrolast cultures at physiological concentrations. Therefore, these suggest that insulin may contribute to the normal regulation of the extracellular matrix. Additional evidence suggests that insulin induced alterations in the extracellular matrix influences the pathogenesis of atherosclerosis. Long term treatment of diabetic and non-diabetic animals with insulin has been shown to result in changes in the arterial wall indicative of atherosclerosis[21-23].

In our study the effect of insulin and IGF-I on the expression of extracellular matrix mRNA in human skin fibroblasts was examined using Northern blot and slot-blot hybridization. In the Northern blot anlysis, ^{32}P labeled pro α1(I) collagen, pro α 1(III) collagen, fibronectin and GAPDH cDNA probes hybridized with each mRNAs specifically. Pro α (I) collagen and pro α 1(III) collagen revealed two mRNA transcripts, which size were 5.8-kb, 4.8-kb, and 5.5-kb, 4.8-kb, and fibronectin and GAPDH revealed one transcript, each size was 8.0-kb and 1.4-kb in insulin or IGF-I treated normal skin fibroblasts. There was no change in it's size, which indicates there was no alteration of it's quality.

Slot-blot hybridization showed that the amounts of pro α1(I) collagen, pro α1(III) collagen, fibronectin mRNAs both in insulin or IGF-I treated normal skin fibroblasts significantly increased compared with untreated normal skin fi-

broblasts. Especially, insulin caused more enhancement of pro α1(I) collagen, pro α1(III) collagen and fibronectin mRNA steady state levels than IGF-I.

Our results show that insulin and IGF-I stimulate collagen formation in skin fibroblasts at physiological concentrations. Therefore these demonstrate that insulin can modulate the expression of the extracellular matrix gene. These data suggest that insulin can directly affect the extrocellular matrix during the wound repair and healing process of diabetic skin diseases, such as NLD. Also, it suggests that long term and high dose treatment of insulin may contribute to the development of diabetic sclerotic skin chang and atherosclerosis or accelerate thoe diseases that accompany the diabetic state in humans. Further studies are needed to clarify the pathhogenesis and underlying mechanism of insulin.

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