THE ROLE OF REACTIVE OXYGEN SPECIES ON UVA-INDUCED AGING OF DERMAL COLLAGEN

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진피 콜라겐의 노화에 대한 활성산소와 자외선의 영향

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Abstract

Considerable interest has been generated in age-related non-enzymatic glycosylation and crosslinking of collagen in view of its extracellular nature, and its long biological half-life. The effects of UVA, which penetrates deep in dermis, and reactive oxygen species(ROS) on age-related changes of dermal collagen were studied. The amount of nonenzymatic glycosylation, fragmentation, and crosslinking of collagen were monitored from the mixtures of Type I collagen from calf skin and glucose, irradiated by UVA, with or without scavengers of ROS. At both high and low glucose dosages, non-enzymatic glycosylation was not affected by UVA irradiation. At high glucose dosage, however, glycosylation was reduced by the scavengers of superoxide radical and singlet oxygen, but not by hydroxyl radical scavengers. Fragmentation was increased by UVA and decreased by all ROS scavengers. Crosslinking was also enhanced by UVA, and heffectively blocked crosslinking. Superoxide radical and singlet oxygen, which were produced by autoxidation of glucose independently to UVA, may

encounter the initial phase of glycation. ROS generated from Amadory compounds by UVA enhanced fragmentation and crosslinking. Hydroxyl radical was thought to be a major ROS affecting crosslinking. These results suggest that UVA and ROS are able to enhance age-related structural changes of collagen, as affecting many other tissue and cellular components

Introduction

Aging process is characterized as being progressive and irreversible under usual condition. Among the most characteristic and definite age changes are changes in mechanical properties of tissues. With age, there is a loss of elasticity in skin, arteries, lungs, and joints(1). Mechanical properties of organs are largely determined by the connective tissues. The state of fibrous proteins of connective tissue, e.g., collagen and elastin, would be expected to play a role in age-related alterations in mechanical properties of organ. Collagen is the most abundant protein in the body and skin. Furthermore, collagen has little turnover and is, thus, good candidate for progressive alteration.

Skin from elderly individuals contains thickened, clumped collageneous material and shows some thickened collagen fibers. Skin collagen becomes less soluble in acid, less digestible by collagenase, less expandable, and more resistant to heat denaturation with advancing age and diabetes melitus(2, 3). Several age-related chemical changes have been described in skin collagen. The molar ratio of glycosylated

hydroxylysine residues to unsubstituted hydroxylysine residues have been reported to increase with age in human skin(4). A reducible fraction from human dermal collagen, which may represent a lysine-carbohydrate condensation product, has also been reported to increase with age(5). Maillard demonstrated the reaction between reducible sugars and amino acids(6), and this reaction was thought to participate in age-related collagen changes(7). The amount of non-enzymatically bound glucose associated with insoluble human skin collagen has been shown to increase as a function of age(8). The age-related increase in glycosylation of human skin collagen may play a role in crosslinking of collagen and the decreased solubility of collagen with increasing age was to be caused by progressive crosslinking. It is noteworthy that skin samples of subjects with diabetes melitus had significantly more insoluble, as well as more glycosylated insoluble, and highly glycosylated collagen(8,9). And several complications of diabetes melitus resemble the general characteristics of aging which occur in collagen rich tissues(10).

Wolff and coworkers(11) demonstrated that glycosylation and fragmentation of proteins were mediated by active species generated through autoxidation of sugar, and they termed "autoxidative glycosylation". Mizunaki and Satto(12) showed that ROS might mediate Maillard reaction. Pathak and coworkers(13) demonstrated the possibility that ROS may participate in collagen crosslinking. They reported that UV enhanced collagen crosslinking *in vitro*, and singlet oxgen quenchers reduced the effect of UV.

The purpose of present work was to investigate the role of each ROS

and UVA on age-related structural changes of dermal collagen *in vitro.*

Materials and Methods

Materials Type I collagen from calf skin, glucose, mannitol, tetrakis-N,N,N′,N′(2-pyridylmethyl)ethylene diamine(TPEN), 1,4-diazabicyclo [2,2,2]octane(DABCO), cyanogen bromide(CNBr), cimetidine, oxalic acid, 5-hydroxymethylfurfural(5-HMF), sodium azide, thiobarbituric acid(TBA), formic acid, and acetonitrile were purchased from Sigma Chem., St. Louis. All other chemicals were of reagent grade.

In vitro *glycosylation of collagen* The mixtures of Type I collagen from calf skin(2 mg/ml) and glucose(0, 50 or 200 mM) in phosphate buffered saline with or without scavengers of ROS were irradiated by UVA(140 mJ/day), and incubated at 37℃ for 4 weeks. After 0, 3, 7, 14, and 28 days of incubation(0, 0.5, 1, 2, and 4J UVA), aliquotes were removed to determine non-enzymatic glycosylation, fragmentation, and crosslinking. Scavengers used were TPEN-Fe(10μ M) and cimetidine-Cu (10μ M) for superoxide radical, azide(100 mM) and DABCO(100 mM) for singlet oxygen, and mannitol (200 mM) for hydroxyl radical. Table 1 lists the experiments

Table 1 Experiments conducted in this study

	glucose (mM)	scavenger	UVA (mJ/day)
1	0	-	-
2	50	-	-
3	50	-	140
4	50	mannitol (200 mM)	140
5	50	azide (100 mM)	140
6	50	DABCO (100 mM)	140
7	50	TPEN-Fe (10μ M)	140
8	50	cimetidine-Cu (10μ M)	140
9	200	-	-
10	200	-	140
11	200	mannitol (200 mM)	140
12	200	azide (100 mM)	140
13	200	cimetidine-Cu (10μ M)	140

conducted in the study. All experiments were done in triplicate.

Determination of nonenzymatic glycosylation After incubation period, the collagen was recovered by centrifugation, washed repeatedly in distilled water to remove unreacted glucose, and lyophilized. Approximate 3-4 mg pieces of lyophilized material were added to 1 ml of 0.5 M oxalic acid. Hydrolysis was carried out for 1 hour in an autoclave at 121 ℃ and 1.2 Kg/cm^2(14). Ketoamine bound carbohydrates were measured according to the method of Trueb *et al.* (15) with some modifications. 400μ l of 40 % trichloroacetic acid were added to collagen hydrolysates, followed by 500μ l of 50 mM thiobarbituric acid. The color was developed by incubation at 40℃ for 45 min, and the absorption was measured at 433 nm using Beckman DU7500 spectrophotometer. All results were expressed as nmole 5-HMF/mg protein, relative to a standard of 5-HMF which was carried through the same procedure.

Protein was measured by Lowry method(16).

Determination of fragmentation Fragmentation was determined by protein measurement. Unprecipitated collagen fragment in the supernatants after centrifugation was quantified by Lowry method.

CNBr fragmentation and SDS/PAGE Collagen crosslinking was measured by the method of Kent *et al*(17) with modification. 300μl of each sample was added to 700μl formic acid. 20μl of CNBr(1 g/ml acetonitrile) was added, and mixtures were incubated in screw-cap bottles at 37℃ for 18 hours. Samples were dialyzed against 0.125 M Tris-Cl buffer pH 6.8(containing 0.2 % SDS and 0.2 % glycerine). CNBr peptides were examined by 8 % sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS/PAGE) with 4 % stacking gel.

Results and Discussion

Non-enzymatic glycosylation At high dose of glucose(200 mM), glycosylation reached its maximum level at 14 days of incubation, and at low glucose level(50 mM), significant increase of 5-HMF content was not detected. At both cases, UVA has no accelerating effect(Figure 1). Superoxide radical and singlet oxygen scavengers greatly reduced non-enzymatic glycosylation under high glucose level, which was about 50% reduction after 28 days, but hydroxyl radical scavenger was ineffective (Figure 2). It means that superoxide radical and singlet oxygen possibly mediate non-enzymatic glycosylation *in vitro,* but these oxygen species

were generated independently with UVA. Autoxidative glycosylation, suggested by Wolff and coworkers(11), might be the case of this reaction. They found that simple sugars are shown to autoxidize, under physiological condition, forming organic free radical, superoxide radical, and hydroxyl radical(18). It should be confirmed by testing the effects of ROS by the same experiments without UVA. *In vivo* situation, however, ROS were generated by UVA through the action of photosensitizers or by UVA-induced inflammatory reactions in the skin (19, 20). Therefore, non-enzymatic glycosylation of dermal collagen was thought to be stimulated indirectly by UVA.

Fragmentation The effects of UVA on fragmentation, measured as a protein content in the supernatants after centrifugation, are shown in Figure 3. At high glucose concentration, fragmentation reached maximum at 14 days and UVA showed no alteration. At low glucose concentration, however, the reaction rate was very low without UVA, and UVA enhanced fragmentation to the level of high glucose concentration. The protective effects of scavengers against UVA-induced fragmentation were tested at low glucose concentration(Figures 4-6). It was shown that all tested scavengers reduced the effect of UVA until 14 days of incubation and, after 28 days, the fragmentation reached to its maximum level. Electrophoresis results showed that low molecular weight fragments were formed by glucose even at low concentration, and UVA enhanced the fragmentation. These fragments were disappeared by all scavengers tested. Azevedo *et al*(21), reported that Amadory compounds, as a result of non-enzymatic glycosylation of protein, are

able to generate superoxide radical. We think that Amadory compounds possibly act as photosensitizer, and ROS produced by Amadory compounds and UVA may cause collagen degradation.

Crosslinking Crosslinked high molecular weight material was detected by SDS/PAGE. Like fragmentation, crosslinking was enhanced by UVA under low glucose concentration. Hydroxyl radical scavenger effectively protected collagen from UVA-induced crosslinking(Figures 7). Therefore, hydroxyl radical was thought to play a major role in crosslinking. In this case, hydroxyl radical was also thought to be generated from Amadory compounds by the action of UVA. Yamamoto and Ishiwatari(22) proposed a mechanism of crosslinking from casein and glucose via Maillard reaction. According to their hypothesis, proteins react with reducible sugar to form Amadory compounds, after then proteins are cleaved and produce fragments, followed by polymerization. They suggested crosslinking was occured by Miallard reaction, as same as initial glycosylation. It was possible that crosslinking was the product of Maillard reaction of primarily glycosylated basic amino acid residues and free basic amino groups in protein molecules. From our results, however, initial glycosylation was mediated by superoxide radical and singlet oxygen, while crosslinking was enhanced by hydroxyl radical.

Conclusion

Followings were concluded, for the effects of UVA and ROS on age-related collagen changes *in vitro,* that

1. non-enzymatic glycosylation was mediated by superoxide radical and singlet oxygen, which were generated by autoxidation of glucose, independently from UVA irradiation,
2. ROS, produced from Amadory compounds by UVA, caused protein fragmentation, and
3. crosslinking, possibly occurred via Maillard reaction, was resulted mainly from the action of hydroxyl radical which was also generated from Amadory compounds by UVA.

According to our results, it is certain that ROS and UVA play a crucial role in the aging of dermal collagen.

요 약

Collagen은 세포 외에 존재하며, 분자의 수명이 매우 긴 단백질이므로, 노화와 관련된 비효소적 당화의 대상으로 많은 관심이 모아지고 있다. 본 연구에서는 진피 collagen의 비효소적 당화와 분해 및 가교결합에 대한 자외선(UVA)과 활성산소종들의 영향을 검토하였다. 암소 피부로부터 얻은 collagen과 glucose의 혼합물에 몇가지 활성산소 소거제를 첨가하고 UVA를 조사하여 비효소적 당화, 단백질 분해, 가교결합의 정도를 관찰하였다. 비효소적 당화는 자외선에 의해 증가되지 않았으며, 수퍼옥사이드 라디칼과 일중항 산소의 소거제에 의해서는 감소하였으나, 히드록시 라디칼 소거제에 의

해서는 변화가 없었다. 단백질 분해와 가교결합은 자외선에 의해 증가되었으며, 분해는 세가지 활성산소의 소거제 모두에 의해 감소되었으나, 가교결합은 특히 히드록시 라디칼 소거제의 영향이 컸다. 즉 수퍼옥사이드 라디칼과 일중항 산소는 자외선과는 관계 없이 당의 자동산화에 의해 발생되어 당화로 인한 변화의 초기 단계에 관여하고, 반응의 뒷부분에서는 당단백질과 자외선에 의해 만들어진 히드록시 라디칼이 주된 역할을 하고 있다. 상기의 결과로부터 활성산소종들과 자외선은 다른 조직 및 세포물질들을 손상시키듯이 노화와 관련된 collagen 변화를 증가시키는 것을 알 수 있었다. 이는 자외선 차단제와 함께 활성산소 소거 물질들을 외용도포하는 것이 태양광선의 해로운 영향으로부터 진피를 보호하는데 도움이 될 수 있음을 의미한다.

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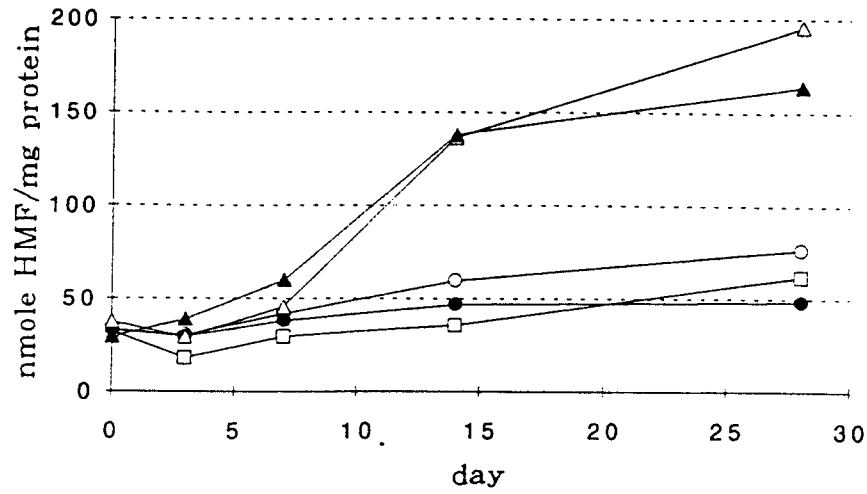

Figure 1. Effect of glucose and UVA on non-enzymatic glycosylation

□ without glucose and UVA; ○ 50 mM glucose;
● 50 mM glucose, irradiated; △ 200 mM glucose;
▲ 200 mM glucose, irradiated.

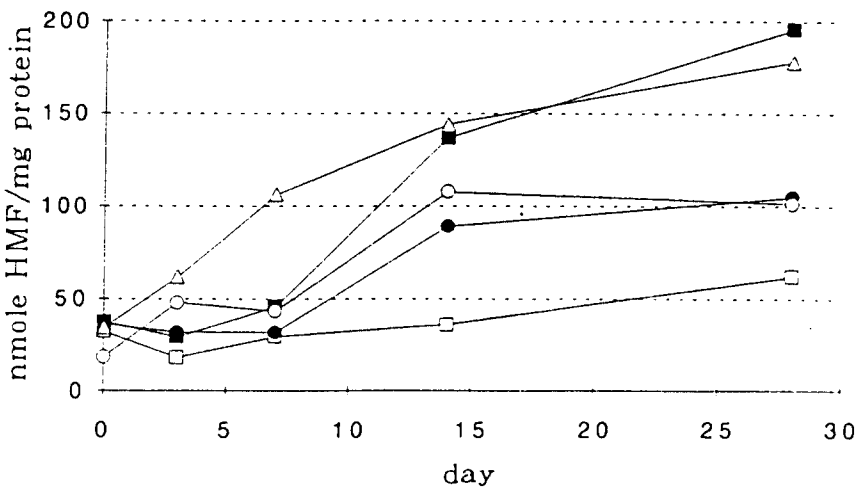

Figure 2. Effect of ROS on non-enzymatic glycosylation in the presence of 200 mM glucose

□ without glucose; ■ no scavenger;
▯ cimetidine-Cu; ● azide; △ mannitol

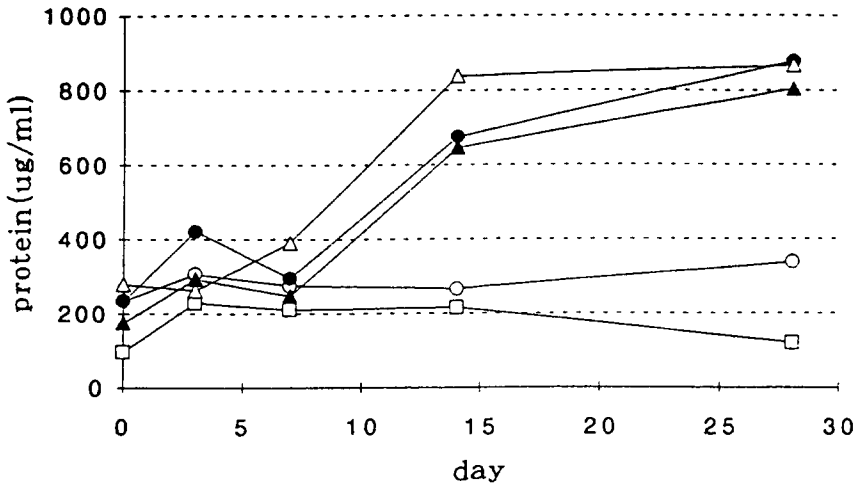

Figure 3 Effect of UVA on collagen fragmentation

□ without glucose and UVA; ○ 50 mM glucose;
● 50 mM glucose, irradiated; △ 200 mM glucose;
▲ 200 mM glucose, irradiated.

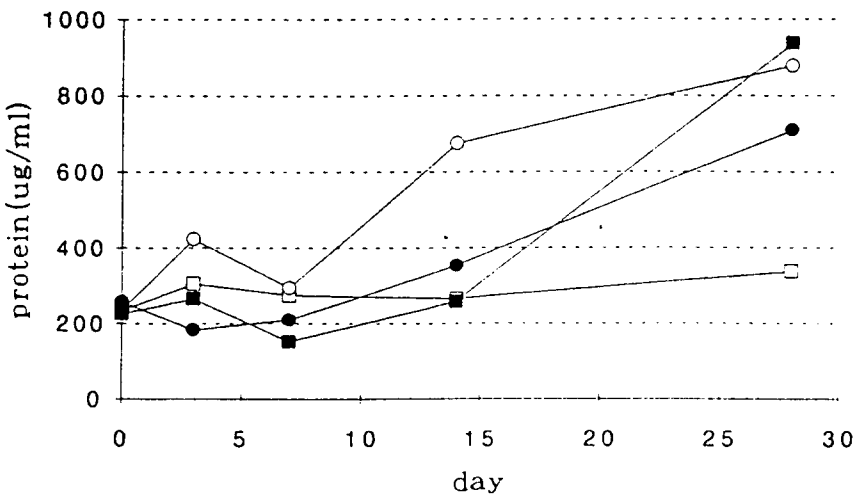

Figure 4 Effect of superoxide radical on fragmentation in the presence of 50 mM glucose

□ unirradiated; ○ irradiated; ■ irradiated with TPEN-Fe
● irradiated with cimetidine-Cu

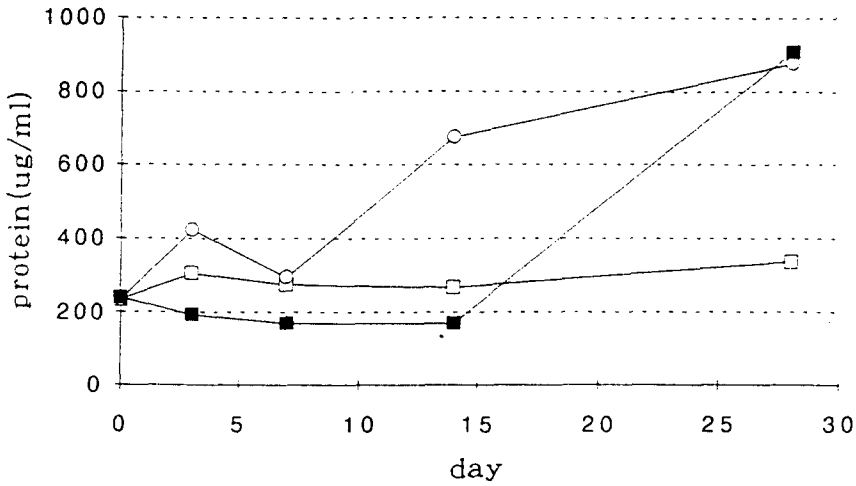

Figure 5 Effect of hydroxyl radical on fragmentation in the presence of 50 mM glucose

□ unirradiated; ○ irradiated; ■ irradiated with mannitol

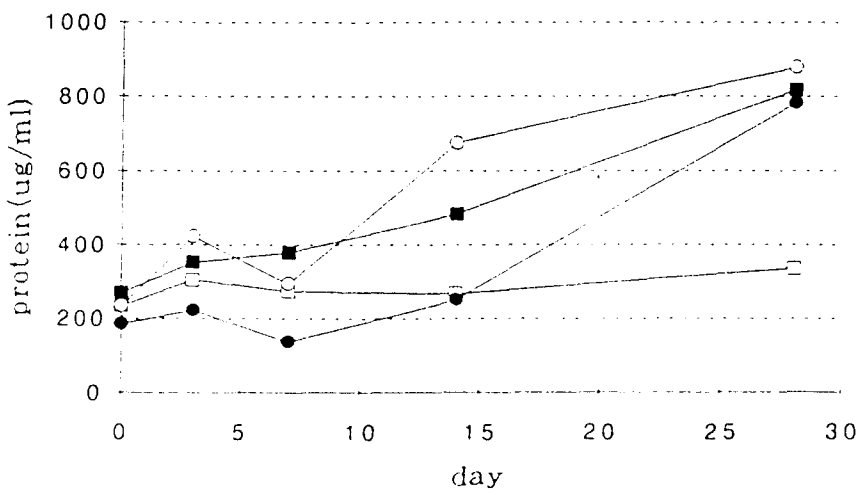

Figure 6 Effect of singlet oxygen on collagen fragmentation in the presence of 50 mM glucose

□ unirradiated; ○ irradiated; ■ irradiated with DABCO

● irradiated with azide

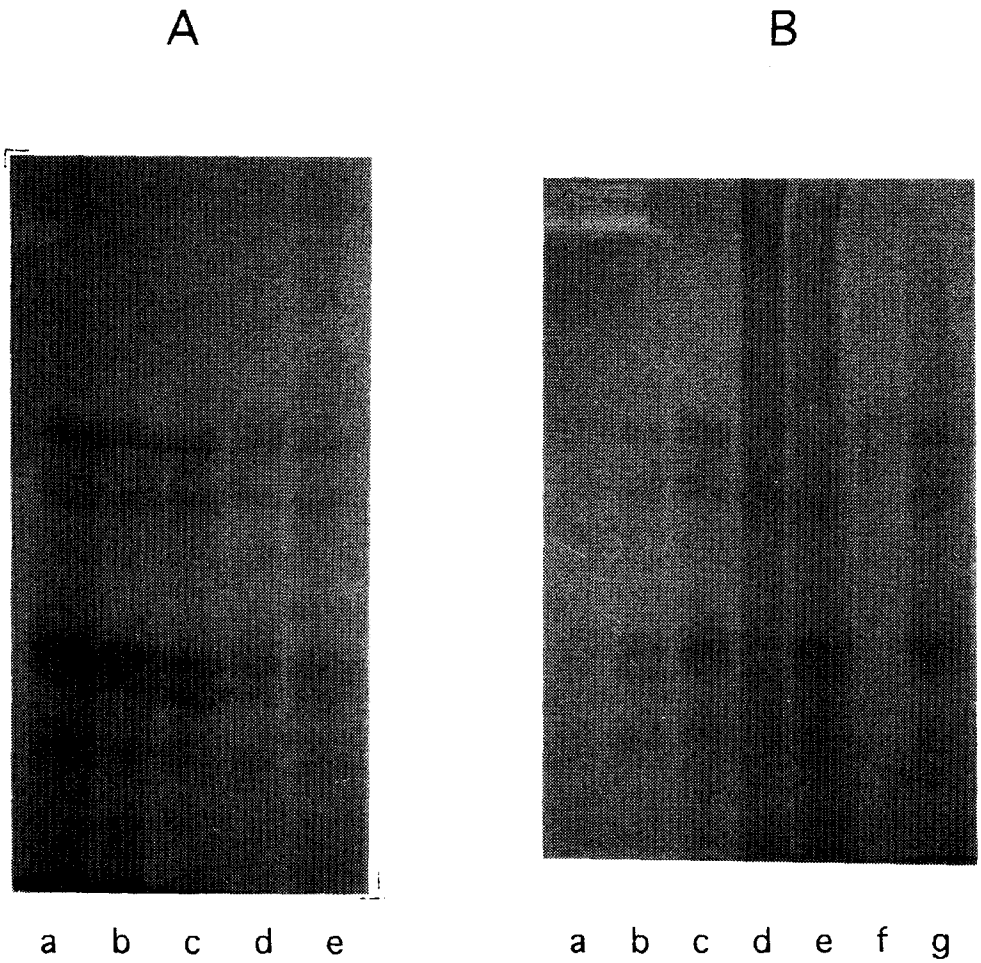

Figure 7. Crosslinking of collagen by glucose and UVA
A. collagen incubated at 50 mM glucose, UVA-irradiated, at various incubation periods(a;0, b;3, c;7, d;14, and e;28 days)
B. collagen treated same as A., with ROS scavengers (c;mannitol, d;azide, e;TPEN-Fe, f;DABCO, and g;cimetidine-Cu). a;without glucose and UVA. b;50 mM glucose, unirradiated