The Effect of UV-A and Reactive Oxygen Species on Glycosylation and Fragmentation of Calf Skin Collagen

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Non-enzymatic glycosylation and fragmentation of collagen molecule were investigated by irradiating Ultraviolet A (UV-A) with or without scavengers of reactive oxygen species (ROS) in the presence of glucose. Non-enzymatic glycosylation was increased by UV-A at high concentration of glucose. It was reduced in the presence of the scavengers of superoxide radical and singlet oxygen, but not reduced in the presence of hydroxy radical seavenger. Fragmentation of collagen was increased by UV-A, but it was desreased in the presence of all ROS scavengers tested. Superoxide radical and singlet oxygen produced by autoxidation of glucose without UV-A may encounter the initial phase of glycosylation. Data presented here suggest that UV-A affects only on the fragmentation process, but all ROS except hydroxy radical act on both processes. It appears that hydroxy radical dose not act on the glycosylation process.

Introduction

Several age-related chemical changes have been described in the skin collagen, which is a major extracellular protein in skin tissue. The representative chemical change for aging of skin is reported to be collagen crosslinking[1]. But the reaction mechanism of crosslinking still remains to be understood. The processes of glycosylation and fragmentation were proposed as the presteps for crosslinking of collagen[2]. The molar ratio of glycosylated hydroxylysine residues to unsubstituted hydroxylysine residues has been reported to be increased with age in human skin[3]. Maillard described the reaction between reducible sugars and amino acids[4] and this reaction has been thought to be participated in age-related structural changes of collagen[5]. The amount of glucose bound to human skin collagen nonenzymatically has been shown to be increased as a function of age[6]. The age-related increase of glycosylation of human skin collagen may play a role in crosslinking of collagen. Highly glycosylated collagens which are extremely insoluble are found in the skin of diabetes[1]. Wolff *et al.*[7] demonstrated that glycosylation and fragmentation of proteins were mediated by active molecular species generated by autoxidation of sugar. There are reports suggesting that ROS may mediate Maillard reaction[8], and UV-A light enhances collagen crosslinking *in vitro* and singlet oxygen quenchers reduce the effect of UV[9].

In the present paper we investigated the role of each ROS and UV-A in glycosylation and fragmentation of bovine collagen *in vitro*..

Experimental

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

Glycosylation of Collagen. The mixture of Type I collagen from calf sikn (2 mg/ml) and glucose (0.50 or 200 mM) in phosphate buffered saline (pH 7.4, 0.05 M) with or without scavengers of ROS was irradiated by UV-A (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 UV-A), aliquots were removed to determine non-enzymatic glycosylation and fragmentation.

Scavengers used were TPEN-Fe (10 μM) and cimetidine-Cu (10 μM) for superoxide radical, azide (100 mM) and DABCO (100 mM) for singlet oxygen, and mannitol (200 mM) for hydroxy radical. Table 1 lists the experiments conducted in the study. All experiments were done three times.

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 of lyophilized material was 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}.[10] Ketoamine bound carbohydrates

Tabla 1. Experiments Conducted in This Study

No.	Glucose (mM)	Scavenger	UV-A (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 (10 μM)	140
8	50	Cimetidine-Cu (10 μM)	140
9	200	–	–
10	200	–	140
11	200	Mannitol (200 mM)	–
12	200	Azide (100 mM)	–
13	200	Cimetidine-Cu (10 μM)	–

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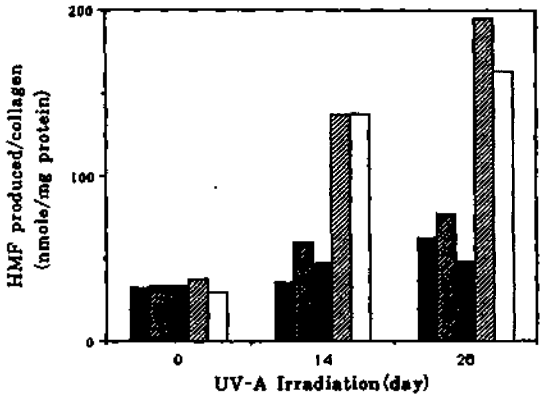

Figure 1. Effect of glucose and UV-A on non-enzymatic glycosylation. ■ without glucose and UV-A; ■ 50 mM glucose; ■ 50 mM glucose, irradiated; ▨ 200 mM glucose; □ 200 mM glucose, irradiated.

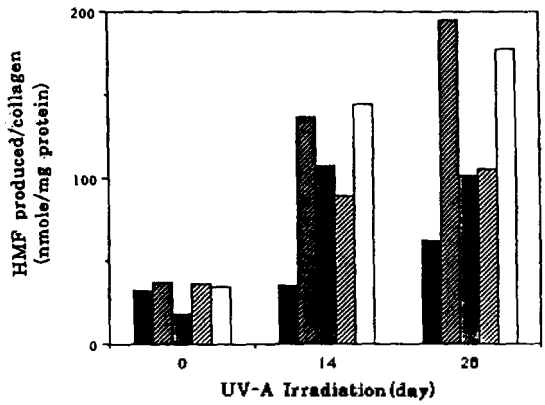

Figure 2. Effect of ROS on non-enzymatic glycosylation in the presence of 200 mM glucose. ■ without glucose; ■ no scavenger; ■ cimetidine-Cu; ■ azide; □ mannitol.

were measured according to the method of Trueb *et al*[11] with some modifications. 400 μl of 40% trichloroacetic acid was added to collagen hydrolysates, followed by 500 μl of 50 mM thiobarbituric acid. The color was developed by incubation at 40℃ for 45 min., and the absorbance was measured at 433 nm (λ_{max} of 5-hydroxymethylfurfural (5-HMF) produced from Amadori compound) using Beckman DU 7500 spectrophotometer.

Determination of Fragmentation. Fragmentation was determined by protein measurement of the unprecipitated collagen fragment in the supernatant after centrifugation and the amount was quantified by Lowry method[12].

Results and Discussion

The most acceptable mechanism for collagen crosslinking in glucose medium has been proposed as three steps; glycosylation, fragmentation and polymerization (crosslinking)[13]. In the present paper, we report how the two processes (glycosylation and fragmention) were affected by UV-A.

Figure 1 shows that significant glycosylation of collagen occurs upon long incubations (14-30 days) at high dose of glucose (200 mM), but no significant increase of glycosylation

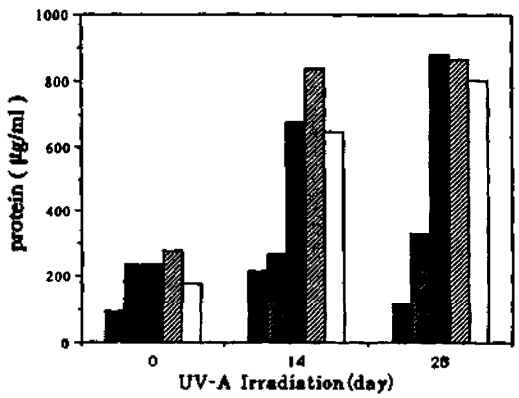

Figure 3. Effect of UV-A on collagen fragmentation. ■ without glucose and UV-A; ■ 50 mM glucose; ■ 50 mM glucose, irradiated; ▨ 200 mM glucose; □ 200 mM glucose, irradiated.

is detected at low level of glucose (50 mM). In both cases, UV-A did not affect glycosylation. However, when we added scavengers (cimetidine and azide) for superoxide radical and singlet oxygen, respectively, great reduction of glycosylation (50% after 28 days) was observed (Figure 2).

In contrast, hydroxy radical scavenger (mannitol) was ineffective. Considering that sensitizers are required for the generation of ROS with light, the generations of these two ROS (superoxide radical and singlet oxygen) must be independent of UV-A, becuase there are no sensitizers in the reaction mixture. At the moment it is not clear how these two ROS are produced independently with UV-A. But it is certain that these two ROS act for the non-enzymatic glycosylation in the presence of 200 mM glucose. The possible interpretation can be presented by autoxidative glycosylation suggested by Wolff *et al.*[7]. Mizubnaki *et al.*[8] found that simple sugars were shown to be autoxidized under physiological conditions to form organic free radicals, superoxide radical and hydroxy radical. However, ROS can be generated *in vivo* by UV-A through the action of endogeneous photosensitizers or by UV-A induced inflammatory reactions in the skin[14]. From our results we can say that non-enzymatic glycosylation of collagen *in vitro* is induced indirectly by UV-A.

The effects of UV-A on the fragmentation of collagen were measured by the protein concentration in the supernatant after centrifugation (Figure 3). At high concentration of glucose, significant enhancement of fragmentations was observed at 14 days after incubation and no UV-A effect on the fragmentation was appeared in this case (Figure 4) However, in the case of low concentration of glucose, drastic enhancement of fragmentation was occurred by UV-A (Figure 5). The inhibitory effects of scavengers on the UV-A induced fragmentation were tested at low glucose concentration (Figure 6). As shown in the figures (Figures 3-6), all kinds of scavengers reduced the effect of UV-A until 14 days of incubation, but the inhibitory effects of scavengers on the fragmentation were disappeared at 28 days of incubation and fragmentation approached the maximum level. Summarizing the results, two questions are evoked: 1) glucose-concentration dependent UV-A effect, and 2) incubation-time dependent inhibition effect of scavengers. Azevedo *et al.*[15] reported

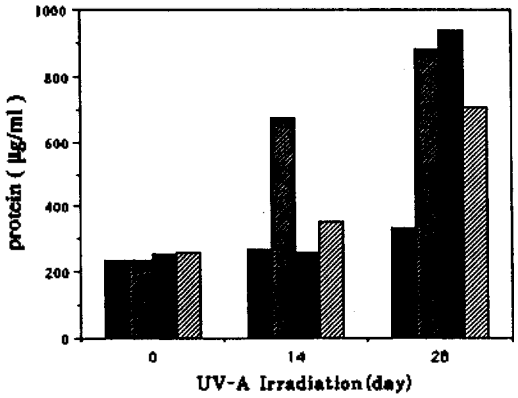

Figure 4. Effect of superoxide radical on fragmentation in the presence of 50 mM glucose. ■ unirradiated; ■ irradiated; ■ irradiated with TPEN-Fe; ▨ irradiated with cimetidine-Cu

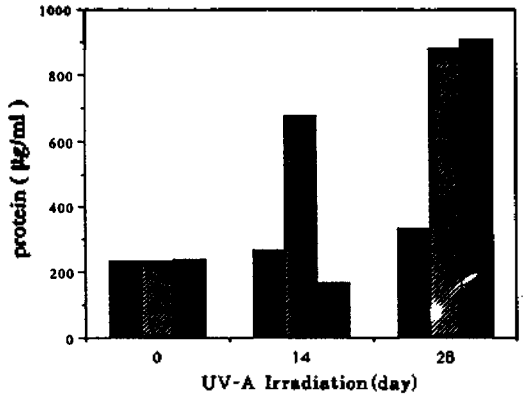

Figure 5. Effect of hydroxy radical on fragmentation in the presence of 50 mM glucose. ■ unirradiated; ■ irradiated; ■ irradiated with mannitol.

that Amadori compounds are able to generate superoxide radical. If Amadori compounds can act as a photosensitizer, irradiation of UV-A can generate photosensitizer to produce ROS even at low concentration of glucose and fragmentation reactions can be induced by these ROS. The fact that no UV-A effect appears at high concentration of glucose may be explained by the saturation effect of ROS produced by autoxidation of glucose. Nonetheless, the second question can not be answered at the moment and further studies are required to be solved.

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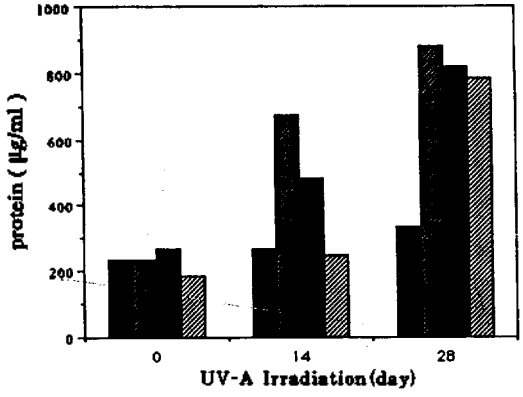

Figure 6. Effect of singlet oxygen on collagen fragmentation in the presence of 50 mM glucose. ■ unirradiated; ■ irradiated; ■ irradiated with DABCO; ▨ irradiated with azide.

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