Repression of Fibroblast Migration into the Collagen Gel Incubated with Glucose Is Due to Glycation-mediated Cross-linking of Collagen

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Abstract: The chemical properties of reconstituted collagen fibrils were changed by prolonged incubation with a high concentration of glucose. The amounts of lysine and arginine residues in the collagen gels decreased after incubation with 0.56 M glucose for two weeks or more, suggesting that glycation of the residues had occurred. CNBr treatment of collagen incubated with glucose yielded polypeptides migrating as bands with lower mobilities on SDS-PAGE than expected for those obtained from collagen not incubated with glucose. Extraction with 0.5 M acetic acid in the presence or absence of pepsin left a greater amount of insoluble materials in the case of glucose-incubated collagen. These results suggest that the glycation of polypeptides eventually led to the formation of covalent cross-links. Interactions between fibroblasts and the collagen gel were examined to see whether the biological activity of collagen gel was changed by the glycation. Cell attachment, spreading, and proliferation on the glycated collagen gel were essentially unaffected, but cell migration from the surface into the collagen gel was markedly inhibited. The cross-linking of collagen gel with NaIO4 also inhibited the cell migration into the gel, but did not affect proliferation. These observations suggest that extensive glycation leads to the cross-linking of collagen and that cross-linking of collagen, regardless of its chemical nature, inhibits cell migration.

Key words: collagen, fibroblast, glycation, migration, cross-linking

INTRODUCTION

Proteins in tissues may be modified in vivo through reaction with high concentrations of glucose, such as may be formed in diabetics. Long-lived proteins (e.g., collagen and lens crystallin) could become substantially glycated, and consequently their physiological functions could be altered or impaired.

The reaction of glucose with proteins was first outlined by Maillard in 1912. The α- or ε-amino groups in proteins react nonenzymatically with the aldehyde groups of reducing sugars to yield Schiff bases, which subsequently afford complex compounds including fluorescent substances through Amadori rearrangement, and cross-linking. Such a sequence of reactions is called a Maillard reaction. The mechanism has not been fully elucidated owing to the highly complex structures of the intermediates and final products. Such structural changes may impair the physiological functions of the proteins, but little work has been done on the relation between structural change and functional deficiency of particular components, perhaps because it is believed that most of the modified proteins will be eventually metabolized and thus leave no significant consequences.

Skeletal structures of organs such as bone, tendon, dermis and cornea contain type I collagen fibrils as a major architectural component. Recently, many reports on type I collagen as a substrate for cultured cells have indicated that it is a potent regulator of cellular functions, including adhesion, proliferation, morphology, migration, the secretion of macromolecules and metabolic activities, particularly when fibroblasts are cultured within type I collagen gel. Such activities of type I collagen gel can be affected by modification of the collagen, e.g., by cross-linking. Indeed, we reported that fibroblast-like cells in vivo migrated into a region occupied by newly formed collagen gel, but did not appear in a region occupied by pre-cross-linked collagen fibrils.

A few reports have appeared on the effect of reducing sugars such as glucose and ribose on the properties of collagen. In the present study, we examined how the incubation of collagen gel with glucose changes the chemical properties of collagen, as well as the ability of collagen gel to act as a cell substrate, in terms of the cell attachment, spreading, proliferation, and migration.

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MATERIALS AND METHODS

Preparation of collagen gel
A solution of pepsin-treated type I collagen in dilute HCl (pH 3.0) at a concentration of 3 mg/ml was purchased from Nitta Gelatin Company (Osaka). A collagen-medium solution (18 ml) was obtained by rapidly mixing 6 ml of collagen solution (3 mg/ml), 3 ml of 3X concentrated phosphate-buffered saline (PBS) containing sodium bicarbonate, penicillin and streptomycin, and 9 ml of PBS, and 2 ml aliquots were placed on 35-mm plastic dishes. The dishes thus prepared (final collagen concentration of 1 mg/ml) were incubated for gelation to occur in a 5% CO2 incubator for 24 h.

Incubation with glucose
Two ml of PBS containing glucose at twice the desired concentration was poured onto the collagen gel in each dish, followed by incubation at 37°C in a humidified CO2 incubator for a period from 1 day to 1 week. When the incubation was continued beyond this time, the PBS containing glucose at the predetermined concentration was renewed at one week. After incubation, the collagen gels were washed eight times with 8 ml of the PBS prior to chemical analysis of collagen, or with 8 ml of Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceutical Company) containing 10% FBS for analysis of cell activities.

Amino acid analysis
The collagen gels incubated with glucose were spun down by centrifugation at 1X10^4 rpm for 10 min. A portion of the sediment was hydrolyzed with 6 M HCl at 105°C for 24 h. Amino acid content was determined with a Hitachi L-8500 amino acid analyzer.

CNBr treatment, acetic acid extraction and pepsin digestion
The collagen gels incubated with glucose were spun down by centrifugation at 1X10^4 rpm for 10 min. A portion of the sediment was suspended in 70% formic acid and subjected to CNBr treatment at 30°C for 24 h. The reaction was stopped by adding 10 volumes of cold water, followed by lyophilization. Another portion of the sediment was stirred in 0.5 M acetic acid or treated with 10 µg pepsin /ml in 0.5 M acetic acid for 2, 6 or 22 h at room temperature. After centrifugation at 1X10^4 rpm for 10 min, the sediments were lyophilized as insoluble collagen and quantified by measuring the content of hydroxyproline.

NaIO4 treatment
Freshly prepared collagen gel was incubated in two ml of PBS containing 0, 0.25, 0.50 or 0.75 mM NaIO4 at 37°C for 15 h. The NaIO4 solution was removed and the gel was incubated with 0.02 g/ml glycine solution, pH 7.5, at 37°C for 3 h. The gel was washed eight times with 8 ml of PBS for chemical analysis of collagen in the gel, or with 8 ml of Dulbecco’s modified Eagle’s medium containing 10% FBS for examination of effects of the collagen gel on cell activities.

Cell culture
Fibroblasts were isolated from human foreskin of a 0-year-old male (HF-0), as described in the previous reports. Fibroblasts at the 19th population doubling level were used throughout the experiments. For the experiments on cell adhesion, spreading, proliferation and migration, 1X10^6 cells suspended in 10% FBS were placed on the surface of collagen gel. Morphological appearance of the cells was evaluated from photographs taken at 6 h after starting incubation at 37°C.

Determination of cell migration into collagen gel
The number of migrated cells was estimated after 3 days of incubation at 37°C by the method described below. The cells that had migrated deeper than 100 micrometers from the gel surface were counted within a defined area of 5 mm^2 in five separate regions with a phase-contrast microscope and expressed as the mean ± S.D. essentially according to Schor et al.? The results were compared with those obtained by Schor’s original method, in which it is required to distinguish migrated cells from elongated cells with the main body situated on the gel but with some processes penetrating into the gel. The two different methods gave essentially the same results (data not shown). The present method has the advantages of taking a shorter time for measurement and of giving lower S.D. values (see Results section).

RESULTS
Change in amino acid composition of collagen caused by incubation with glucose
Table 1 shows the amino acid compositions of collagen incubated with and without glucose. In the case of incubation with a low glucose concentration (0.06 M) or incubation with a high glucose concentration (0.56 M) for a short time (less than 7 days), no change in the amino acid composition was detected. On the other hand, prolonged (14 or 21 days) incubation at a high glucose concentration (0.56 M) caused a reduction in

Table 1: Contents of lysine and arginine residues of collagen after incubation with glucose.

<table>
<thead>
<tr>
<th>Glucose incubation time (M)</th>
<th>(days)</th>
<th>Lys</th>
<th>Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>1</td>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td>0.06</td>
<td>21</td>
<td>23</td>
<td>50</td>
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<tr>
<td>0.56</td>
<td>1</td>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td>0.56</td>
<td>7</td>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td>0.56</td>
<td>14</td>
<td>21**</td>
<td>45**</td>
</tr>
<tr>
<td>0.56</td>
<td>21</td>
<td>21**</td>
<td>45**</td>
</tr>
</tbody>
</table>

Collagen gel that had been incubated with glucose was hydrolyzed with 6 M HCl at 105°C for 24 h for amino acid analysis. Control is collagen not incubated with glucose.

* Significantly different from controls; p < 0.05.
** Significantly different from controls; p < 0.01.
contents of lysine and arginine residues. Since the sample was hydrolyzed in 6 M HCl at 105°C without pretreatment with a reducing reagent such as NaBH₄, Schiff bases, if present, would have been cleaved to reform the original amino and carbonyl groups.

**SDS-PAGE analysis of CNBr digests of collagen incubated with glucose**

Incubation of reconstituted collagen fibrils with a low glucose concentration, regardless of duration, did not change the SDS-PAGE band patterns of CNBr digests of collagen fibrils. However, collagen incubated with a high glucose concentration showed weaker bands of low-molecular polypeptides and strong bands of higher-molecular polypeptides. This suggests that the Maillard reaction of glucose with collagen may eventually lead to cross-linking of the polypeptides in reconstituted collagen fibrils (Fig. 1).

**Dissolution in acetic acid, in the presence or absence of pepsin, of collagen fibrils incubated with glucose**

Materials insoluble in acetic acid in the presence or absence of pepsin were obtained from glycated collagen gel. Collagen gel incubated with a high glucose concentration (0.56 M) for 3 weeks was insoluble in 0.5 M acetic acid even in the presence of pepsin, whereas collagen gel without glucose incubation was completely soluble. Incubation at a high glucose concentration resulted in increased amounts of materials insoluble in 0.5 M acetic acid, with or without pepsin (Table 2).

**Effect of incubation of collagen gel with glucose on cellular activities**

Cell adhesion to collagen gels was not affected by incubation of collagen fibrils with glucose under the conditions used in the present study (data not shown). Cell spreading on reconstituted collagen gel was slightly depressed by incubation of gel with a high concentration of glucose (0.56 M) for a prolonged period such as 3 weeks (Fig. 2). Cell proliferation on collagen gels incubated with glucose was essentially unaffected (data not shown). The most striking effect of incubation of reconstituted collagen gel with glucose was seen on fibroblast migration into the gel. Incubation of reconstituted collagen gel with glucose at a concentration above 0.28 M for 3 weeks inhibited the migration of cultured fibroblasts into the collagen gel (Fig. 3). When the concentration of glucose was high (0.56 M), an incubation time of only 1 week caused a decrease in the cell migration (Fig. 4). Fibroblasts that had migrated into the collagen gel showed an elongated morphology, regardless of whether the gel had been incubated with a high glucose concentration or not (data not shown).

**Effect of NaI₅ treatment**

The cell migration into the gel was depressed upon treatment of collagen gel with 0.75 mM NaI₅ to an extent similar to that caused by glycation (Fig. 5). The cell proliferation was not affected (data not shown). The SDS-PAGE band pattern of CNBr digests of collagen gel treated with 0.50 mM NaI₅ showed polypeptide bands with lower mobilities on SDS-PAGE, suggesting the formation of covalent cross-links (data not shown).

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**Table 2** Dissolution in acetic acid, in the presence or absence of pepsin, collagen incubated with glucose.

<table>
<thead>
<tr>
<th>Dissolution in acetic acid</th>
<th>Treatment time (h)</th>
<th>% of insoluble materials</th>
<th>Control</th>
<th>Collagen incubated with glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>79.9±7.3</td>
<td>86.0±11.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>76.2±4.5</td>
<td>85.2±4.2*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>11.2±1.2</td>
<td>86.0±6.0**</td>
<td></td>
</tr>
<tr>
<td>acetic acid with pepsin</td>
<td>2</td>
<td>89.6±5.8</td>
<td>102.7±17.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>48.5±3.1</td>
<td>33.4±6.8**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>6.9±1.8</td>
<td>63.6±3.4**</td>
<td></td>
</tr>
</tbody>
</table>

Collagen gel that had been incubated with 0.56 M glucose for 3 weeks was stirred in 0.5 M acetic acid or treated with 10 μg/ml pepsin in 0.5 M acetic acid for 2, 6, and 22 h at room temperature. The control is collagen not incubated with glucose. * Significantly different from controls; p<0.05. ** Significantly different from controls; p<0.01.
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Fig. 2. Phase-contrast micrographs of human fibroblasts on collagen gel treated with different concentrations of glucose.

Collagen gel was incubated for 1 day with glucose at a concentration of (A) 0, (B) 0.006, or (C) 0.56 M and for 3 weeks with glucose at a concentration of (D) 0, (E) 0.006, or (F) 0.56 M. After incubation, the gels were washed with culture medium without glucose. Fibroblasts were plated on collagen gel (HF-O, PDL-19, at a density of $1 \times 10^5$ cells/dish). Photographs were taken at 6 h of culture. Bar, 200 micrometers.

Fig. 3. Effect of incubation of collagen gel with glucose on the migration activity of human fibroblasts into the gel.

Collagen gels were incubated with an indicated concentration of glucose for 1 day or 3 weeks. The gels were washed, then fibroblasts were plated (HF-0, PDL-19, at a density of $1 \times 10^5$ cells/dish) onto the surface of the gels and cultured for 3 days. The number of migrated cells was estimated by counting cells located deeper than 100 micrometers from the gel surface. Significantly different from incubation with 0 M glucose for 3 weeks: $^* p<0.05$, $^{**} p<0.01$. No significance was not seen in experiment of incubation with glucose for 1 day.

Fig. 4. Effect of the incubation time of collagen gel with 0.56 M glucose on the migration activity of human fibroblasts into the gel.

Collagen gels were incubated with 0.56 M glucose for 1, 3, 7 or 21 days. Fibroblasts (HF-0, PDL-19) were plated onto the surface of gels, and then cultured for 3 days. The number of migrated cells was determined. Significantly different from no incubation with glucose: $^* p<0.05$, $^{**} p<0.01$.

Fig. 5. Effect of NaIO$_4$ treatment of collagen gel on migration activity of human fibroblasts into the gel.

Collagen gel was treated with 0, 0.25, 0.50 or 0.75 mM NaIO$_4$. Fibroblasts (HF-0, PDL-19) were plated onto the surface of gels, and then cultured for 3 days. The number of migrated cells was determined. Significantly different from incubation without NaIO$_4$: $^* p<0.05$, $^{**} p<0.01$.

DISCUSSION

Previously reported electron microscopic observations of cross-sections of glycated tendons incubated with ribose showed an increase in the fibril diameter, increased density of fibril packing, fibril fusion, and distortion of fibrillar packing$^{15-18}$. Glycation causes cross-linking between collagen molecules in collagen fibrils, and this in turn alters the physio-chemical properties of the fibrils. We studied the effect of incubation with glucose on reconstituted collagen fibrils in order to address three questions. What chemical or physicochemical properties of collagen fibrils were changed by incubation with glucose? What interactions between fibroblasts and
collagen were affected by the chemical change of collagen fibrils? How could these changes be related?

Incubation of proteins with glucose results in glycation of the amino groups of proteins. The present study confirmed that reconstituted collagen fibrils became covalently cross-linked by glycation after incubation with glucose, as expected from previous reports. We previously showed that the cellular activities of fibroblasts depend greatly on the presence of collagen fibrils in the environment. Cross-linking of collagen fibrils repressed cell migration into the gel, but had much less effect on cell spreading and proliferation. Cell attachment was least affected by glycation-mediated cross-linking. Why was only the cell migration strongly affected by glycation-mediated cross-links? It is noteworthy that cell migration from the surface into collagen gel was also suppressed by the treatment of collagen with NaIO₄, a cross-linking agent (Fig. 5), while cell proliferation on the gel was not affected. We previously reported that fibroblast-like cells migrated into collagen gel reconstituted in situ from an injected solution, beginning at 1 day after injection into rat dermis, while migration into pre-cross-linked collagen did not occur up to 7 days after injection. The observations can be consistently explained by assuming that cell migration into collagen gel is retarded by cross-linking, regardless of the cross-link structure. Why was cell migration impaired by cross-linking of collagen fibrils? Howard et al. reported that cellular contraction of collagen fibril gels was inhibited by glycation. We obtained essentially the same results (data not shown). Since cell attachment to, spreading on, and detachment from the collagen gel were not affected by extensive cross-linking, some unique feature of the interaction of cell with collagen gel must be involved in cell migration and collagen gel contraction.

In collagen gel contraction, change in cell shape or cell movement appears to be strongly related with collagen fibril rearrangement. Collagen fibrils begin to be “tugged” or “arranged” by the fibroblasts at 12 h of culture just when the cells start to change shape. In cell migration into the gel, change in cell shape and cell movement must inevitably cause changes of the collagen fibril arrangement. How easily the collagen fibril arrangement can be changed might well depend on the degree of cross-linking. We speculate that flexibility of collagen fibrils is important for any phenomenon that requires rearrangement of collagen fibrils. This would include cell migration into or through collagen gel, as well as collagen gel contraction. Such flexibility would be impaired by cross-linking.

References
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