Age-Related Changes in the Pyridinoline Content of Guinea Pigs Cartilage and Achilles Tendon Collagen

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Summary Age-related changes of pyridinoline, a mature cross-link of collagen fibers, in tissues of guinea pigs which cannot synthesize L-ascorbic acid (AsA) were investigated. Male guinea pigs, 2 weeks old, were fed a commercial diet until they were 40 weeks old. Based on the data of body weight gain, it is assumed that guinea pigs require 10 weeks to reach maturity. The content of AsA in serum, adrenals, and liver of the animals increased with growth, then decreased after 8–10 weeks. The pyridinoline content in cartilage and tendon collagen was very low in 2-week-old animals, whereas it increased markedly with growth. After 10 weeks, the pyridinoline tended to decrease in cartilage collagen; however, it was not changed in tendon collagen. The age-related changes of pyridinoline content in guinea pigs cartilage were similar to those of humans—increasing with growth and decreasing after adolescence.

Key Words pyridinoline, cartilage, Achilles tendon, collagen, guinea pigs, AsA, mature cross-link, age-related changes

Collagen cross-links play an important role in providing strength and consistency to the tissues (1). It is generally agreed that stability of collagen fibers increases with advancing age (2). Pyridinoline, a mature cross-link which is stable and non-reducible, is derived from two hydroxyallysine and one hydroxylysine residues of collagen fibrils (3, 4). Fujimoto et al. isolated and characterized pyridinoline in Achilles tendon collagen. They proposed that pyridinoline is formed through dehydrated condensation of the reducible immature cross-link, dehydrodihydroxylysinoonorleucine, with hydroxyallysine (4–6).

Recently, urinary excretion of pyridinoline has been demonstrated (7–11). In contrast to the wide distribution of collagen, pyridinoline is mainly present in bone and cartilage, but in lesser amounts in other connective tissues. Therefore, the urinary pyridinoline is assumed to be potentially more useful than urinary hydroxyproline as a marker of the catabolism of collagen fibers in skeletal tissues. The pyridinoline content in collagen of fetal or newborn animals is very low and
increases markedly with growth in rats or humans (2).

The changes in the content of pyridinoline during maturity varied with species and tissues. In the case of rats, the pyridinoline content in cartilage continued to increase gradually up to 17 months. On the other hand, it began to decrease after about 20 years of age in human tissues (2). It is well known that rats synthesize AsA and do not require it in their diet; however, humans cannot synthesize AsA because they lack the liver enzyme, L-gulono-γ-lactone oxidase. AsA is required for collagen synthesis, mainly because it acts as a cofactor of prolylhydroxylase and lysylhydroxylase in the post-translationally modifying reaction of procollagen (I2–I4). In the biosynthesis of collagens, the enzymatic hydroxylations of proline and lysine are necessary for the production of a stable extracellular matrix and cross-links in the fibers, respectively (I5). Therefore, we hypothesized that AsA would affect the formation of pyridinoline directly or indirectly since the contents of AsA in human tissues gradually decrease during aging (I6–I9). In this study, we investigated the age-related changes in the content of pyridinoline and AsA in tissues of guinea pigs which cannot synthesize AsA.

MATERIALS AND METHODS

Animals and diets. Fifty-four male albino guinea pigs (Hartley strain; Sankyo Labo Service Corporation, Inc., Tokyo Japan), 2 weeks old (about 200 g), were fed commercial diet (Clea Japan Inc., Tokyo, Japan, 100–150 g/kg body wt.) for 38 weeks until they were 40 weeks old. At the beginning of the experiment, the animals were divided into nine groups (2, 3, 4, 8, 10, 12, 16, 24, 40 weeks).

Assay of alkaline phosphatase activity. Alkaline phosphatase (ALP) activity in serum was determined by using the alkaline phosphata-B-Test kit (Wako Pure Chemical Ind., Osaka, Japan). The serum was added to the reaction mixture containing a substrate, p-nitrophenyl phosphate, and incubated at 37°C for 15 min. The optical density of the reaction mixture was read on a spectrophotometer at a wavelength of 405 nm.

Determination of AsA. AsA in the tissues and serum was extracted with 5% metaphosphoric acid and determined by high-performance liquid chromatography (HPLC; solid phase, LiChrosorb-NH2; mobile phase, 0.01 M phosphate buffer, pH 3.3; flow rate, 0.7 ml/min, detector, UV 254 nm).

Preparation of tissues for analysis of hydroxyproline and pyridinoline. Hyaline cartilage of the ribs and Achilles tendon samples from guinea pigs were cut into small pieces, washed in saline and defatted with methanol : chloroform (1:2). The tissue samples were hydrolysed in 6 N HCl in sealed tubes at 110°C for 24 h and evaporated to dryness under vacuum.

Determination of collagen. The collagen was measured from its hydroxyproline content, assuming that the hydroxyproline content was 0.11 mol per mol collagen (I2I). Hydroxyproline contents in the resulting hydrolysates were determined by the method described by Woessner et al. (I2).
Determination of pyridinoline. The pyridinoline standard was prepared from bovine Achilles tendon collagen according to a method by Fujimoto et al. (23). Pyridinoline contents of the hydrolysates were determined by HPLC method described in our previous paper (24); solid phase, Inertsil ODS-2; mobile phase, 0.1 M phosphate buffer, pH 3.5 and acetonitrile (25:75) containing SDS 1 g/liter EDTA 25 mg/liter; flow rate, 0.1 ml/min. For the HPLC analysis, the dry residues of the hydrolysates were dissolved in 2 ml of water and filtered through a chromatodisc (pore size 0.45 μm). Ten microliters of each sample was injected. The eluate was monitored by fluorescence with excitation at 295 nm and emission at 395 nm. Pyridinoline content was determined by a comparison with an external standard and expressed as mol per mol collagen.

RESULTS

Growth data of guinea pigs in this experiment, which included from 50- to 80-week-old animals, are shown in Fig. 1. They showed rapid increase in weight from 2 to 10 weeks old. The body weight gain after 10 weeks until 50 weeks became less than that from 2 to 10 weeks, then a slight retardation of body weight started after 50 weeks. Because some animals over 50 weeks became weak and died, only body weight is shown.

Organ weights (g/100 g body wt.) in animals for 2–40 weeks are shown in Table 1. Liver, lung, and heart weights increased gradually with growth, then did not change after 10 weeks. Significant enlargement of adrenals was evident with growth.

The measurement of the ALP activity in serum is a biochemical index to follow

![Graph](attachment:body_weight_graph.png)

Fig. 1. Changes in body weight of guinea pigs.

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Table 1. Changes in tissue weight of guinea pigs.

<table>
<thead>
<tr>
<th>Week</th>
<th>(No.)</th>
<th>Liver</th>
<th>Adrenals</th>
<th>Lung</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>(5)</td>
<td>8.32±0.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08±0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.47±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>(6)</td>
<td>12.25±0.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.09±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.68±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.90±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>(5)</td>
<td>10.37±0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.72±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.93±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>(5)</td>
<td>14.21±1.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.17±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.27±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>(5)</td>
<td>23.31±2.38</td>
<td>0.23±0.02</td>
<td>3.09±0.12</td>
<td>1.59±0.10</td>
</tr>
<tr>
<td>12</td>
<td>(6)</td>
<td>23.38±0.74</td>
<td>0.25±0.02</td>
<td>2.63±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.67±0.09</td>
</tr>
<tr>
<td>16</td>
<td>(5)</td>
<td>22.78±1.86</td>
<td>0.43±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.98±0.15</td>
<td>1.85±0.03</td>
</tr>
<tr>
<td>24</td>
<td>(5)</td>
<td>23.80±0.19</td>
<td>0.49±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.68±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.60±0.18</td>
</tr>
<tr>
<td>40</td>
<td>(6)</td>
<td>24.88±1.76</td>
<td>0.78±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.36±0.08</td>
<td>2.44±0.10</td>
</tr>
</tbody>
</table>

1 Values: M±SE. The number of guinea pigs is given in parentheses. Significantly different from the value of 10 weeks at a (p<0.001), b (p<0.01), c (p<0.05), respectively.

Fig. 2. Serum alkaline phosphatase activity. Alkaline phosphatase activity of the serum was determined by using the alkaline phosphaB-Test kit.

the health condition in animals. Figure 2 shows the ALP activity of guinea pigs; the activity was relatively low during aging.

Table 2 reveals the age-related changes of AsA in serum, adrenals, and liver of guinea pigs. Although liver and adrenals weights increased greatly from 8 to 10 weeks, there was no significant difference between AsA concentrations at 8 and 10 weeks. That is, the content of AsA in adrenals and liver of guinea pigs increased with growth, then decreased during aging. Moreover, in serum, it was also the
Table 2. Concentration of AsA in serum, adrenals, and liver of guinea pigs.

<table>
<thead>
<tr>
<th>Age (week)</th>
<th>Serum (mg/100 ml)</th>
<th>Adrenals (mg/100 g)</th>
<th>Liver (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.87±0.11</td>
<td>73.2±15.5</td>
<td>18.1±4.5</td>
</tr>
<tr>
<td>3</td>
<td>1.33±0.31</td>
<td>66.7±6.0</td>
<td>21.3±4.2</td>
</tr>
<tr>
<td>4</td>
<td>1.26±0.48</td>
<td>94.6±8.7</td>
<td>21.0±3.1</td>
</tr>
<tr>
<td>8</td>
<td>1.52±0.20</td>
<td>151.8±14.7</td>
<td>31.1±9.2</td>
</tr>
<tr>
<td>10</td>
<td>2.37±0.77</td>
<td>177.3±37.4</td>
<td>19.5±2.3</td>
</tr>
<tr>
<td>12</td>
<td>2.02±0.55</td>
<td>131.3±5.9</td>
<td>17.1±1.8</td>
</tr>
<tr>
<td>24</td>
<td>0.86±0.21</td>
<td>132.8±10.1</td>
<td>15.5±0.8</td>
</tr>
<tr>
<td>40</td>
<td>0.47±0.10</td>
<td>62.0±5.3</td>
<td>19.2±2.5</td>
</tr>
</tbody>
</table>

\(^{1}\)Values: M±SE. Significantly different from the value of 10 weeks at c \((p<0.05)\).

Fig. 3. Concentration of collagen in cartilage and tendon as a function of age.

highest level at 10 weeks and gradually decreased with aging. At 40 weeks, the AsA content in serum was as little as 20% of that found in the growing process.

Figure 3 shows the collagen contents per g of wet weight tissue in cartilage and tendon. The content of collagen in both cartilage and tendon of 2-week-old animals was very low. Their contents increased markedly until 15 weeks, and then reached a plateau. This value was approximately 2 fold in comparison with those of 2-week-old animals.
Fig. 4. Concentration of pyridinoline in collagen from cartilage and tendon as a function of age. Pyridinoline content is expressed in mol per mol collagen.

The age-related changes in the pyridinoline content of cartilage and tendon collagens are shown in Fig. 4. The pyridinoline content in cartilage was very low in 2-week-old animals, increased markedly with growth, and tended to decrease after 10 weeks. However, the content in tendon collagen was not changed with growth.

DISCUSSION

The present study indicates that the guinea pig which is unable to synthesize AsA as well as humans, may be an appropriate animal model for evaluating the effect of nutrients on age-related changes of collagen cross-links. The loss of ability of biosynthesis of AsA gave the different characteristics on growth and other physiological indices. Vazer et al. reported that the body weight in rats continued to increase throughout their life span (25). Our results show that male guinea pigs can be divided into two age categories before 50 weeks old: the growing process until 10 weeks and the period of maturity after 10 weeks. This is in agreement with the previous observation that the age of maturation in male guinea pigs is 10 weeks (26). It was also supported by the ALP activity, which is known to be relatively low in the period of maturity.

The other physiological index is AsA content in the tissues. AsA content in serum at 40 weeks was only 20% that found at 10 weeks although the intake of AsA was about 200 and 100 mg/kg body weight/day, respectively. Most studies reported that age-related AsA levels of whole blood, plasma, and leucocytes in human declined with aging (27–29). Our results also showed that age-related changes of
AsA content in serum and tissues of guinea pigs were similar to those of human. Therefore, we confirmed that species that cannot synthesize AsA in vivo lose the content of AsA in tissues with aging even if fed the higher amounts of AsA.

It has been reported that the collagen content in connective tissues gradually increases with aging and mature collagen remains insoluble in either neutral salt or diluted acetic acid (25). The half-life of skin collagen was found to be longer in mature rats than in young ones (60 and 27 days, respectively) (30, 31). We observed that the content of collagen in both cartilage and tendon of 2-week-old animals was very low, but increased with growth. This indicates that the collagen content in these connective tissues of guinea pigs increases during aging.

In contrast, as shown in Fig. 4, the pyridinoline content in cartilage increased markedly with growth and tended to decrease after 10 weeks, although it was very low in collagen of newborn guinea pigs. Moriguchi and Fujimoto (2) reported that the pyridinoline content in fetal or newborn rat and human was very low and increased markedly with growth. They also reported that the pyridinoline content in the rat tissues continued to increase gradually up to 17 months corresponding to the period of the maturity, but in human cartilage began to decrease after 20 years of age. Our results indicate that the pyridinoline content in cartilage of guinea pigs changes with aging in a manner similar to that of humans.

As pyridinoline is one of the predominant cross-links in a mature collagen, pyridinoline formation may be an essential step during the growing process to obtain normal mechanical strength in collagen fibers. The excess formation of pyridinoline in collagen during this period will probably make the tissue stiffer, less soluble and less digestible by enzymes. In guinea pigs, the content of AsA and pyridinoline decreased during maturity as well as it did in humans. In the tissues of rats, such alteration of pyridinoline apparently did not occur (2). These results suggest that the age-related changes in pyridinoline content are influenced by AsA content. Moreover, pyridinoline may reflect an important aspect of aging for both the maturation and the senescence of the connective tissues in guinea pigs.

REFERENCES


samples containing small proportions of this amino acid. Arch. Biochem. Biophys., 93, 440–447.


