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Effect of L-Ascorbic Acid Contents in Tissue on Collagen Synthesis in Guinea Pigs

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Abstract

To clarify the requirement of L-ascorbic acid (AsA) in collagen synthesis, the incorporation of 1-^{14}C-proline into the tissues of guinea pigs and the specific radioactivity ratio (proline/hydroxyproline) in collagen were investigated. Male guinea pigs maintained on the AsA-deficient diet were divided into three groups ; group A (AsA-deficient animals) ; group B (control animals) supplemented with 5mg AsA/day ; group C (high dose animals) with 300mg AsA/day, and orally supplemented with or without AsA for 14 days. Collagen synthesis was estimated by measuring the incorporation of labeled proline into collagen in lung and dorsal skin, and the hydroxyproline contents in lung and skin. The AsA contents in the tissues were determined by high-performance liquid chromatography (HPLC), and serum alkaline phosphatase activity was also measured. The serum alkaline phosphatase activity of AsA deficient group was very low as compared with those of AsA supplemented group. Incorporation of labelled proline into collagen and its specific radioactivity ratio in collagen increased with increasing levels of AsA in the tissues. There was a significantly positive relationship between the levels of AsA and hydroxyproline in the tissues.

Key words : ascorbic acid, collagen synthesis, proline hydroxylation, guinea pigs

INTRODUCTION

Collagen is synthesized from the polypeptide precursor, which is called procollagen, and the synthesis of the collagen molecule depends upon hydroxylation of prolyl and lysyl residues contained in this pre-cursor[1,2]. Procollagen that contains underhydroxylated proline is unstable and does not appear to have a triple helical structure[1]. AsA is required in vitro and in vivo for optimal function of prolyl hydroxylase, hydroxylation of peptidyl proline, and enhancing secretion of procollagen that contains hydroxyproline[2]. Collagen synthesis decreased under AsA deficient conditions due to the inhibited proline or lysine hydroxylation[1-3].

There are many evidences in which AsA contents of tissues in animals are correlated with proline hydroxylation on collagen synthesis[1-4]. Particularly in the wound healing process, the higher contents of AsA in tissues could be effective for the rapid collagen synthesis by increasing the prolyl hydroxylase activity[3]. However, it is still unclear about optimum tissue level of AsA which can affect the maintenance of collagen in intact tissue.

In this study, to get more information about the requirement of AsA on collagen synthesis in intact tissues, the effect of various tissue levels of AsA on the incorporation of 1-^{14}C-proline into the tissues of guinea pigs and the specific radioactivity ratio in collagen were investigated.

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MATERIALS AND METHODS

Reagent

AsA was obtained from Wako Pure Chemical Industries, Ltd. 1-^{14}C-proline was obtained from Amersham Japan Co.

Animals and diets

Male albino guinea pigs (Hartly strain) with initial body weights of about 250g were housed individually in wire cages, and maintained on the AsA-deficient diet.

Water and diets were offered ad libitum through the experimental period. After prefeeding with 5mg AsA/day for 4 days, they were randomly divided into three groups according to the following experimental plans.

Experimental procedures

The animals were divided into three groups. Group A (AsA-deficient animals) were fed AsA-deficient diets for 14 days. Group B (control animals) were orally supplemented with 5mg AsA/day[4], group C (high dose animals) with 300mg AsA/day. AsA was dissolved in distilled water immediately before use and administered orally. The animals were weighed daily. On day 14 of feeding, the animals were intraperitoneally injected with or without 1-^{14}C-proline, and were sacrificed 3 hours later[5]. The liver, lungs and dorsal skin were removed to determine the amounts of AsA and to extract the collagen.

Assay of enzyme

Serum alkaline phosphatase acitivity was determined by using Alkaline Phospha B-Test (Wako Pure Chemical Industries, Ltd.) with p-nitrophenylphosphate as substrate[6].

Determination of AsA

The preparation of tissue samples for determination of AsA was described in a previous report[7]. The concentration of AsA in tissues was determined by HPLC. HPLC conditions were as follows : a Shimadzu LC-5A was used, and the eluate was monitored with a UV detector at 254nm. A stainless steel column (250 x4 mm i. d.) was packed with Unisile-NH_2, and phosphate buffer (pH 3.3, 0.01M NaH_2PO_4/0.01M HPO_3) was used as the mobile phase at a flow rate of 0.7ml/min.

Determination of ^{14}C-proline, ^{14}C-hydroxyproline and hydroxyproline

Collagen was isolated by the method of Barnes et al.[1] The isolated collagen from dorsal skin was hydrolyzed in 6N HCl in a sealed tube at 110° C for 24h. Hydroxyproline in the hydrolysate was determined by the method of Woessner [8].

The Radioactivities of ^{14}C-proline and ^{14}C-hydroxyproline were determined by using HPLC with a radio analyzer (Aloka)[9].

RESULTS AND DISCUSSION

Fig. 1 shows the body weight changes of the guinea pigs in three groups. The growth response of group B (supplemented 5mg/day) was almost equal to the group C supplemented 300mg/day, however, the AsA deficient group A showed a considerable

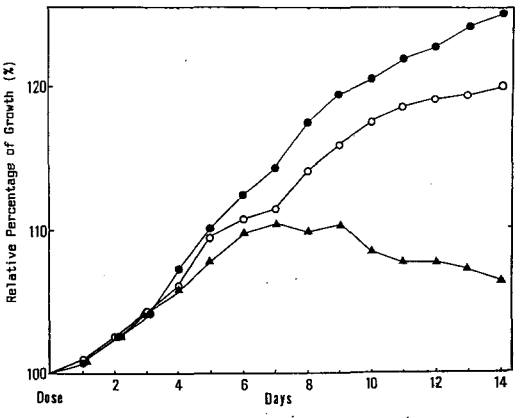

Fig. 1. Changes in body weight of guinea pigs.
Group A (▲), AsA-deficient group ; Group B (○), 5mg AsA supplemented group ; Group C (●), 300mg AsA supplemented group.

reduction in body weight gain after 10 days and some characteristic symptoms of scurvy after 12 days. No statistical significant difference in body weight gain was observed between group B and group C, but the body weight gain in group C showed a tendency to be slightly larger than that of group B after 5 days, probably indicating that the growth was slightly affected by high intakes of AsA used in this study. This result was almost consistent with the observation that 100mg AsA/100g body weight (BW)/day, which was equal to approximately 300~350mg AsA/animal of 300~350g BW/day, had no influence on growth [10,12].

Table 1 shows the activity of serum alkaline phosphatase of all groups. The enzyme activity of AsA-deficient group was significantly lower than those of groups B and C, and the result agreed with the previous reports[4,7] which showed a very low enzyme activity in scorbutic state. On the other hand, there was no significant difference between group B and C in their enzyme activities, indicating that high intakes of AsA gave almost no influence on the value of the enzyme activity.

Table 2 shows AsA contents in liver, lung and skin. The AsA contents in the tissues of AsA deficient animals were low. The amounts of AsA in all the tissues of group C were significantly higher than those of group B. It was reported that AsA levels in some tissues such as kidney, heart, spleen, liver and adrenal glands of the animals administered above 50mg AsA/100g BW remained almost constant and biodegradation products of AsA in urine increased[10]. In this study, it was not certain whether the AsA contents in liver, lung and skin were saturated with the dose of AsA 300mg/day or not. However, it was observed that AsA contents in the skin and lung of group C were about 5 and 3 times larger than those of group B, respectively (Table 2).

Collagen, which is a constituent of connective tissue in every organ in the body, is found abundantly in skin or lung[9]. The process of wound healing is necessary collagen synthesis in a connective tissue scar, and the process could be accelerated by the supplementation of AsA[13-15]. Since the collagen synthesis rate obtained for lung and skin were 2~3 times larger than those observed in the other tissues[9], we expected that the collagen turnover rate in the intact tissues may be affected by the AsA contents in the tissues.

The level of proline incorporation into protein can be considered as a measure of the synthesis of collagen type proteins[11]. Therefore, we determined the incorporation of 1-^{14}C-proline into skin and its radioactivity ratio (proline/hydroxyproline). Table 3 shows incorporation of 1-^{14}C-proline into skin and its specific radioactivity ratio (proline/hydroxyproline). The specific radioactivity ratio in collagen was above 1.0 in the AsA-deficient group, indicating that the incorporation of proline and its hydroxylation were inhibited under the AsA-deficient condition. On the other hand, the specific radioactivity ratio was approximatly 1.0 in both of the the AsA

Table 1. Alkaline phosphatase activity

Group	(No)	Activity (BL unit)
A	(4)**	0.57±0.12*[a]
B	(4)	7.87±0.32[b]
C	(4)	7.65±0.29[b]

*Mean±S.E. **The number of guinea pigs is in parenthesis. Means with the different letter are significantly different at the 0.01 level of significance as determined by the Duncan's multiple range test

Table 2. Ascorbic acid contents in tissues of guinea pigs

Group (No)	Liver	Lung	Skin mg/100g (wet tissues)
A (4)**	0.69±0.42*[a]	1.38±0.01[a]	N.D.[a]
B (4)	3.35±0.86[b]	10.52±0.12[b]	0.55±0.06[b]
C (4)	15.87±0.98[c]	27.10±0.08[c]	2.27±0.97[c]

*Means±S.E. **The number of guinea pigs is in parenthesis. N.D. : Not detected. Means in the same column with the different letter are significantly different at the 0.01 level of significance as determined by the Duncan's multiple range test

Table 3. Incorporation of 1-^{14}C-proline into collagen and its hydroxylation ratio

Group	Hydroxyproline (dpm/g wet weight of skin)	Proline/Hydroxyproline
A	5,971	1.21
B	9,647	0.99
C	18,366	0.93

supplemented groups. The results obtained in this study were consistent with the findings of Barns et al[1]. Interestingly, the ratio in group C showed a tendency to be apparently lower than that of Group B, indicating the proline hydroxylation could be activated by increasing AsA contents in the tissue of group C supplemented with AsA 300mg/day.

Fig. 2 shows a relationship between AsA contents in tissues and hydroxyproline in collagen in the tissues. A considerable positive relationship was found between the AsA levels and hydroxyproline contents in collagen in the tissues. The hydroxyproline contents in collagen of the lung and skin in AsA-deficient groups were lower than those of groups B and C, and the hydroxyproline contents in the collagen of group C supplemented with AsA 300mg/day were higher than those of group B supplemented 5mg/day, which is in agreement with the earlier results as described above. Moreover, it was also considered that the function of AsA-dependent prolyl hydroxylase could still be decreased in animals which did not even show scurvy, but were receiving optimal amounts of dietary AsA.

From these results, it was suggested that the amounts of AsA necessary to maintain normal growth of animals may not be equivalent to the amount required for the various AsA-dependent enzyme. In addition to this, it was also considered that the degree of proline hydroxylation would be markedly susceptable to the AsA contents in tissues. Therefore, a higher level of AsA contents in tissues than those previously thought could contribute to the proline hydroxylation for the maintenance of collagen in the intact tissues as well as for the wound healing process.

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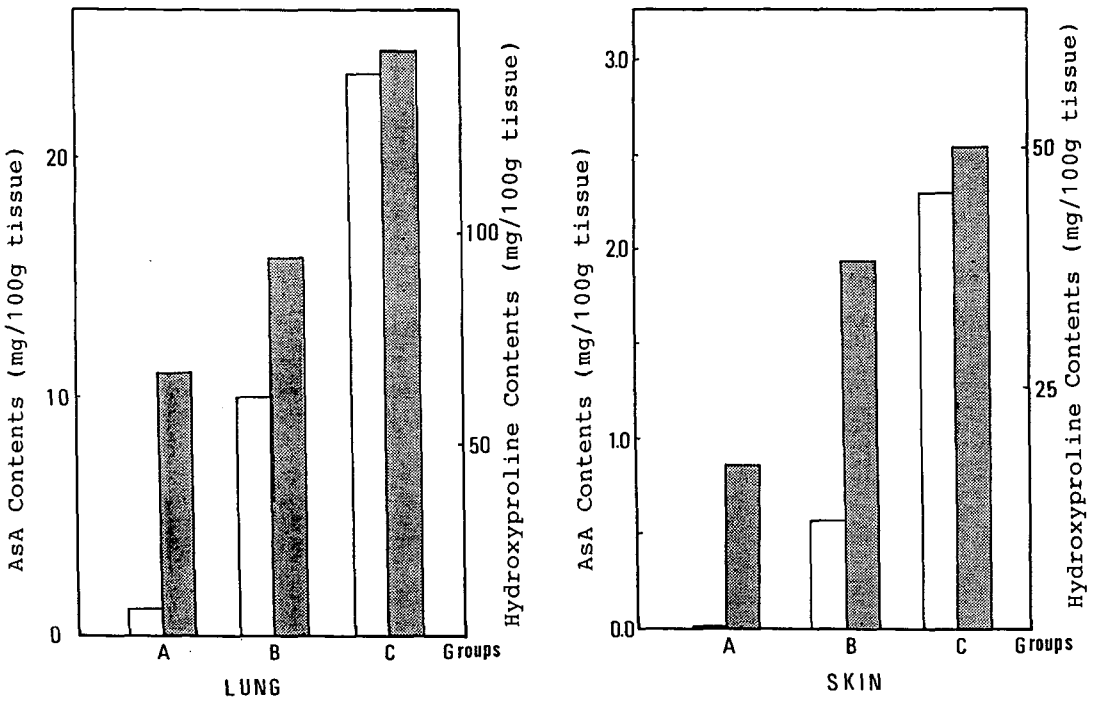

Fig. 2. Ascorbic acid contents in tissues and hydroxyproline contents in collagen.
□ : AsA, ■ : Hyp

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Guinea pig의 조직중 L-Ascorbic acid 함량이 콜라겐 생합성에 미치는 영향

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요 약

L-Ascorbic acid (AsA) 생합성이 불가능한 guinea pig을 실험 동물로 하여 collagen 함량이 높은 조직인 폐 및 피부중의 AsA 함량과 동일조직중 collagen함량이 높은 조직인 폐 및 피부중의 AsA 함량과 동일조직중 collagen의 proline잔기의 수산화율을 조사하여 collagen 생합성에 대한 조직중 AsA의 영향에 대해서 알아보았다. Guinea pig(체중 약 250g)를 AsA 무투여군(A), AsA 5mg/day 투여군(B), 300mg/day 투여군(C)으로 나눠 14일간 사육한 후, 마취하에서 개복하여 복부 대동맥으로부터 채혈함과 동시에 간장과 폐를 적출하였으며, 등부위의 피부를 재취하여 분석용 시료로 하였다. 이들 시료로부터 혈청중 alkaline phosphatase (ALP)활성과 각조직중의 AsA함량, proline 함량 및 그 수산화율, (1-^{14}C) proline 의 incorporation 양을 측정하였다. 그 결과, AsA투여군인 B, C군의 경우 순조로운 체중증가와 함께 혈청중 ALP활성도 정상값을 나타냈으며 현저한 ALP활성 저하가 관찰되었다. 한편, AsA 투여군인 B군과 C군의 경우 유사한 ALP활성값을 나타냈으나, 폐와 피부중의 AsA 함량이 높을수록 (1-^{14}C) proline의 incorporation된 양이 많고 collagen 중의 hydroxyproline함량도 증가하는 것으로 나타나, 조직중의 collagen합성량과 AsA함량과의 사이에는 높은 상관관계가 존재함이 확인되었다.