Biochemical and Molecular Roles of Nutrients

Vitamin C Deficiency in Guinea Pigs Differentially Affects the Expression of Type IV Collagen, Laminin, and Elastin in Blood Vessels

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ABSTRACT Vitamin C deficiency causes morphologic changes in the endothelial and smooth muscle compartments of guinea pig blood vessels. Endothelial cells synthesize the basement membrane components, type IV collagen and laminin, and smooth muscle cells synthesize elastin in blood vessels. Therefore, we examined the possibility that vitamin C deficiency affects the expression of these proteins. Decreased expression of types I and II collagens in other tissues of vitamin C–deficient guinea pigs is associated with weight loss and the consequent induction of insulin-like growth factor binding proteins; thus we also used food deprivation to induce weight loss. Female guinea pigs received a vitamin C–free diet, supplemented orally with ascorbate. Vitamin C–deficient guinea pigs received the same diet but no ascorbate, and the food-deprived group received no food, but were supplemented with vitamin C. Concentrations of mRNAs for basement membrane components and elastin in blood vessels were measured by Northern blotting; overall basement membrane metabolism was assessed by measuring immunoreactive laminin and type IV 7S collagen in serum. Laminin mRNA in blood vessels and serum laminin concentrations were unaffected by vitamin C deficiency. Concentrations of type IV collagen and elastin mRNAs in blood vessels were not significantly affected in moderately scorbutic guinea pigs (0–7% weight loss), but with increased weight loss, type IV collagen mRNA was 57% (P < 0.05) and elastin mRNA was 3% (P < 0.01) of normal values. In food-deprived guinea pigs, type IV collagen mRNA was 51% (P < 0.05) and elastin mRNA was 35% (P < 0.05) of normal. Serum type IV 7S collagen concentrations were 25% of normal in scorbutic guinea pigs with extensive weight loss. The lower expression of type IV collagen and elastin mRNAs in blood vessels may contribute to defects observed in blood vessels during scurvy. J. Nutr. 129: 83–91, 1999.

KEY WORDS: guinea pigs • ascorbate • basement membrane collagen • elastin • laminin

In addition to weight loss, one of the most obvious symptoms of scurvy in guinea pigs is hemorrhaging, especially at the rear knee joint (Chatterjee 1967, Friederici et al. 1966). During severe vitamin C deficiency in humans, there is hemorrhaging under the periosteum at the ends of the long bones of children, and in the joints, skin and gums of adults (Vilter 1967). A combined histologic and electron microscopic study found that there is much less intercellular material in the aortic endothelium of scorbutic guinea pigs (Gore et al. 1965). A histologic examination of blood vessels within and surrounding a subdermal polyvinyl implant in guinea pigs found numerous changes in large and small veins and arteries caused by vitamin C deficiency (Stolman et al. 1961). Endothelial cells were swollen and protruded into the lumen of vessels, and the endothelial membranes separated from the vessel wall, whereas smooth muscle cells lost their spindle shape. An electron microscopic study also found morphologic changes in endothelial cells of blood vessels of scorbutic guinea pigs that included dilated cisternae of the endoplasmic reticulum by vitamin C deficiency (Stolman et al. 1961). Endothelial

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short N-terminal noncollagenous domain plus collagenous sequences. It contains four cysteines and several lysine residues that participate in crosslinking to form an organized structure (Hudson et al. 1993, Kuhn 1994). Laminin is another important structural component of basement membrane that also plays a role in cell attachment, proliferation and differentiation (Timpl and Brown 1994, Yurchenco and O’Rear 1994). Numerous studies indicate that laminin is expressed in blood vessel endothelial cells (Sephel et al. 1996 and references therein). It has three separate polypeptide chains, designated as α, β and γ, that are linked by disulfide bonds to form a cross-shaped molecule. There are several variants of each subunit, but the molecule in blood vessels is composed mainly of α1, β1 and γ1 chains (Yurchenco and O’Rear 1994); recent evidence suggests, however, that a different α-chain may be present (Sephel et al. 1996). Other components of the basement membrane include entactin and heparan sulfate proteoglycans (Yurchenco and O’Rear 1994).

Another major component of blood vessels is elastin, which is synthesized by smooth muscle cells in the tunica media as a soluble precursor referred to as tropoelastin (Oakes 1982, Rosenbloom 1984). Tropoelastin is extensively crosslinked by the copper-requiring enzyme lysyl oxidase in the extracellular space to form insoluble elastin.

Our previous studies (Gosiewska et al. 1994, Peterkofsky 1991, Peterkofsky et al. 1994) have shown that during the first 2 wk of consuming a vitamin C–free diet, referred to as phase I of vitamin C deficiency, guinea pigs grow normally, although vitamin C levels are decreased early. During wk 3 and 4 (phase II), the guinea pigs become anorexic and lose weight. Weight loss is associated with a decrease in the expression of mRNAs for type I collagen in skin and bone, and type II collagen in cartilage, as well as proteoglycan synthesis in cartilage. During this period, there is induction of circulating insulin-like growth factor binding protein-1 and -2 (IGFBP-1 and -2) that inhibit collagen, proteoglycan and DNA synthesis in cultured connective tissue cells by competing with the insulin-like growth factor-I (IGF-I) receptor for binding of the ligand (Gosiewska et al. 1994, Peterkofsky 1991, Peterkofsky et al. 1994). Guinea pigs that are food-deprived for 4 d, but supplemented with vitamin C, show similar effects (Gosiewska et al. 1994, Peterkofsky 1991, Peterkofsky et al. 1994). The induction of IGFBP-1 and -2 appears to explain the lower expression of types I and II collagens in scurbitic and food-deprived guinea pigs (Gosiewska et al. 1994, Peterkofsky et al. 1994). There is only moderate lowering of hydroxylation of proline residues in collagen synthesized during phase II of vitamin C deficiency, which is not correlated with the effects on collagen synthesis (Peterkofsky et al. 1987), and there is no decrease at all during phase I (Kipp et al. 1995). Not all symptoms of vitamin C deficiency, however, can be explained by the weight loss/IGFBP effect. These include defective wound healing (Kipp et al. 1995), iron deficiency and changes in the expression of iron-related proteins (Gosiewska et al. 1996), and lower expression of alkaline phosphatase and osteocalcin in bone (Mahmoodian et al. 1996). The objectives of this study were to determine whether vitamin C deficiency affected the gene expression of laminin, type IV collagen and elastin in blood vessels, or the overall metabolism of basement membrane components, as measured by their levels in serum, and whether such changes, if they occurred, were related to weight loss.

### MATERIALS AND METHODS

#### Animals and diets.

Protocols for obtaining vitamin C–deficient and food-deprived, vitamin C–supplemented guinea pigs are described in detail previously (Gosiewska et al. 1994, Kipp et al. 1995, Peterkofsky et al. 1994) but are summarized here. A pelleted vitamin C–free guinea pig diet was obtained from ICN (Irvine, CA); its composition is shown in Table 1. Female guinea pigs of strain 2, STZ/N, were obtained from the NCI-FCRF animal facility and weighed 148 ± 2 g on arrival. They were fed the ascorbic acid–free diet and were supplemented daily with a 25 g/L solution of sodium ascorbate in 10 g/L sucrose, delivered orally using a sterile plastic transfer pipet, at a dose of 25 mg/100 g body weight. When the guinea pigs were gaining weight at the rate of ~ 7 g/d, a group of guinea pigs was placed on a vitamin C–deficient regimen by continuing the diet, but withdrawing ascorbate supplementation. The control guinea pigs continued to receive vitamin C supplementation. The guinea pigs in the food-deprived regimen were not given any food for 4 d, but were supplemented daily with ascorbate, as described above. All groups had free access to water and were weighed daily.

Vitamin C–deficient guinea pigs began to lose weight after ~ 2 wk of consuming the vitamin C–free diet; they were killed during wk 3 and 4. The percentage of weight loss for vitamin C–deficient guinea pigs was calculated from the difference between weight at the time the guinea pig was killed compared with the highest weight that was attained before weight loss commenced. Control guinea pigs also were killed during that 2-wk time period. During that period, a portion of the control guinea pigs were started on the food-deprivation regimen. For the food-deprived group, weight loss was calculated based on their weight when they were killed compared with their weight at the time food was withdrawn. Our previous studies established that whether weight loss occurs gradually through food restriction (pair-feeding), rapidly through complete food deprivation or as a result of vitamin C deficiency, there is an identical decrease in collagen synthesis in bone and cartilage that is proportional to weight loss (Bird et al. 1986, Chojkier et al. 1983).

The groups of guinea pigs described in this study were used also in our previous studies. Scurbitics from one set (Mahmoodian et al. 1996) lost 0, 4.8, 7, 13.4, 20, 25 and 28% of their body weight during wk 3 and 4 of consuming the vitamin C–free diet. Scurbitic guinea pigs with 0% weight loss were still growing after 16–17 d of consuming the vitamin C–free diet; they were killed during wk 3 and 4. The percentage of weight loss for vitamin C–deficient guinea pigs was calculated from the difference between weight at the time the guinea pig was killed compared with the highest weight that was attained before weight loss commenced. Control guinea pigs also were killed during that 2-wk time period. During that period, a portion of the control guinea pigs were started on the food-deprivation regimen. For the food-deprived group, weight loss was calculated based on their weight when they were killed compared with their weight at the time food was withdrawn. Our previous studies established that whether weight loss occurs gradually through food restriction (pair-feeding), rapidly through complete food deprivation or as a result of vitamin C deficiency, there is an identical decrease in collagen synthesis in bone and cartilage that is proportional to weight loss (Bird et al. 1986, Chojkier et al. 1983).

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### TABLE 1

Composition of ascorbic acid–deficient diet

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/kg diet)</th>
</tr>
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<tr>
<td>Skim milk powder</td>
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</tr>
<tr>
<td>Vitamin-free casein</td>
<td>100</td>
</tr>
<tr>
<td>Ground oats</td>
<td>400</td>
</tr>
<tr>
<td>Ground bran</td>
<td>150</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>80</td>
</tr>
<tr>
<td>Cottonseed oil</td>
<td>50</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>10</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
</tr>
</tbody>
</table>

*1 The prepared diet was obtained from ICN Pharmaceuticals, Costa Mesa, CA.*

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*Abbreviations used: DTT, dithiothreitol; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; NEM, N-ethylmaleimide; PCR, polymerase chain reaction; PMSF, phenylmethyl-sulfonylfluoride; RT-PCR, reverse transcriptase-PCR.*
TABLE 2

<table>
<thead>
<tr>
<th>Probe (species)</th>
<th>Sense 5′</th>
<th>Antisense 5′</th>
<th>PCR product (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>collagen α1(IV) (guinea pig)</td>
<td>gactggctcatggtc</td>
<td>ttcttgctggacca</td>
<td>260</td>
<td>50</td>
</tr>
<tr>
<td>laminin (guinea pig)</td>
<td>caggggttgccatgct</td>
<td>ccagcttggcaaggct</td>
<td>516</td>
<td>55</td>
</tr>
<tr>
<td>elastin (rat)</td>
<td>caggggttgccatgct</td>
<td>ccagcttggcaaggct</td>
<td>471</td>
<td>45</td>
</tr>
</tbody>
</table>

1 The primer pairs correspond to the following cDNA nucleotide bases: rat elastin, 1000–1014 and 544–553; mouse α1(IV), 260–275 and 16–32; mouse laminin, 4333–4350 and 3835–3854.

RT-PCR reaction. The reverse transcriptase reaction was carried out with 1 μg of total RNA from blood vessels or liver in a total volume of 25 μL, as described previously (Gosiewska and Peterkofsky 1995), except that 2 μL of SuperScript II RNaseH-free reverse transcriptase ( Gibco BRL, Gaithersburg, MD), and 1.6 × 10^4 units/L of Rnasin (Promega, Madison, WI) were used. The reaction was terminated by the addition of an equal volume of cold water, and 10 μL of the RT reaction mix was used for the PCR step, carried out in a 100 μL volume as described previously (Gosiewska and Peterkofsky 1995).

Northern blot hybridization. Total RNA or poly(A)^+ RNA was denatured in 370 g/L formaldehyde plus formamide for 15 min at 65°C and electrophoresed on a 12 g/L agarose-formaldehyde gel. The RNA was blotted onto a Magna NT ( MSI, Westboro, MA) membrane with a Turbo-Blotter (Schleicher & Schuell, Keene, NH), and the membranes were prehybridized for 30 min with ExpressHyb hybridization solution (Clontech, Palo Alto, CA) at 68°C. The cDNA probes were labeled with [32P]dCTP (Amersham) by nick translation, and hybridized at 68°C for 1 h with ExpressHyb hybridization solution, as described by the manufacturer. The membranes were washed three times in 2X SSC containing 0.5 g/L SDS at room temperature for 15 min, two times in 0.1X SSC containing 1 g/L SDS at 30°C for 20 min and then exposed to X-ray film. To normalize for the amounts of RNA applied to the membranes, probes were stripped and blots were rehybridized with a 30 base oligonucleotide probe for 28S rRNA (Takeda et al. 1992) that was end-labeled with [35S]dATP (Amersham). Conditions for hybridization with the oligomer in ExpressHyb were those described by the manufacturer and differed slightly from those for cDNA probes. Results were quantitated by densitometric scanning of autoradiograms using the Image program (W. Rasband, NIH) for the Macintosh computer.

Ammonium sulfate precipitation of serum proteins. Partial purification of collagen and laminin fragments in serum was achieved by fractionation with ammonium sulfate at 33% saturation; serum albumin is not precipitated at this concentration. A 200-μL portion of serum was adjusted to contain 2 mmol/L EDTA, 1 mmol/L N-ethylnaleimide (NEM), 0.2 mmol/L phenylmethyl-sulfonylfluoride (PMSF), 0.1 mol/L HEPES, pH 7.2, and 0.4 mol/L NaCl. Half a volume of saturated ammonium sulfate was added to the solution; after a 30-min incubation on ice, the samples were centrifuged at 20,000 × g for 10 min at 4°C. Supernatants were removed and pellets were dissolved in 40 μL of 0.2 mol/L NaCl containing 0.05 mol/L HEPES buffer, pH 7.2.

Collagenase digestion. A 5-μL portion of the ammonium sulfate-precipitated serum proteins was incubated for 30 min at 37°C in a final volume of 10 μL containing 7.5 mmol/L NEM, 4.2 mmol/L CaCl2, 12 mmol/L Tris-HCl, pH 7.6, with or without 2.5 μg of purified bacterial collagenase, as described previously (Peterkofsky et al. 1995). In some experiments, samples were reduced with dithiothreitol (DTT) before collagenase digestion. An equal volume of twice concentrated SDS-PAGE sample buffer, with or without 20 mmol/L DTT, was added to the samples, which were heated at 100°C for 5 min, cooled and then fractionated by SDS-PAGE.

Immunoblotting with antibodies to type IV collagen and laminin. Ammonium sulfate–fractionated serum proteins were electrophoresed on 6 or 10% SDS-polyacrylamide gels, and immunoblotting was carried out as described previously (Mahmoodian et al. 1996), except that polyclonal antibodies against human placental type IV collagen (Calbiochem, La Jolla, CA), and human placental laminin (Life Technologies, Gaithersburg, MD) were used. Immunoreactive proteins were detected using the ABC system from Vector Laboratories (Burlingame, CA) with biotinylated second antibody and peroxidase substrate, according to the manufacturer's instructions. Human placental, pepsin-extracted type IV collagen (Collaborative Biomedical Products, Bedford, MA) and laminin (Life Technologies) were used as standards. Protein concentrations in serum were measured with the BCA protein assay, using reagents and a bovine serum albumin standard from Pierce (Rockford, IL).

Statistical analysis. The Graph Pad Prism statistical analysis program (version 2.0, San Diego, CA) was used to analyze values for collagen and elastin mRNA concentrations in blood vessels of a single set of experimental and normal guinea pigs. The significance of
RESULTS

Scorbutic symptoms. Guinea pigs fed a vitamin C–free diet lost body weight at varying rates during wk 3 and 4, as described previously (Gosiewska et al. 1994, Kipp et al. 1995); they also exhibited hemorrhaging at the knee joints during that period. Guinea pigs that had lost 20% of their body weight after 72 and 96 h of food deprivation with vitamin C deficiency (Fig. 2A, lane 6) was less intense in the 72-h sample compared with the control (Fig. 2B, lane 1, 2), whereas 10 ng was in that range but was not adequate for visualization (Fig. 2B, lane 3). Another analysis with 25–100 ng of RNA from scorbutic guinea pig blood vessels indicated that 25 ng was still within a concentration-dependent range of response and was adequate for visualization (Fig. 2B, lanes 5 and 6). Therefore, 25 ng of RNA from blood vessels of normal, scorbutic and food-deprived guinea pigs was used for analysis of the 516-bp cDNA fragment. The results suggested that there was no significant difference in laminin γ1 mRNA in samples from scorbutic (Fig. 3, lanes 4, 5) or food-deprived (Fig. 3A, lanes 7–10) guinea pigs compared with those from controls (Fig. 3A, lanes 1–3, and 6); this was confirmed by densitometry of the gels (data not shown).

Measurement of immunogenic laminin fragments in serum has been used as an index of changes in laminin metabolism in liver fibrosis (Hirayama et al. 1996) and in vascular diseases (Burgmann et al. 1996). Ammonium sulfate precipitates of serum proteins were separated by 6% SDS-PAGE, and immunoblotted with antibody against laminin. The human pepsin–extracted laminin standard contained 170- and 190-kDa fragments after reduction (Fig. 3B, lower panel, lane 6). The immunoreactive laminin fragments in serum from normal, scorbutic and food-deprived guinea pigs (Fig. 3B) were similar in size to the standard pepsin fragments, but the 170-kDa species was the major component. There was no apparent effect on total serum laminin levels during vitamin C deficiency (Fig. 3B, upper panel); this conclusion was confirmed by scanning densitometry of both fragments on this blot and others (data not shown). The major 170-kDa fragment was unaffected by food deprivation, although the 190-kDa band was less intense in the 72-h sample compared with the control (Fig. 3B, lower panel, lane 4).

A: Collagen α1(IV) 1

B: Laminin γ1 Chain 1

differences between values for experimental groups compared with normal values was evaluated using a two-tailed Student's t test; they were considered significantly different with P < 0.05. When variance between groups was different, as determined from F-values, the data for those groups also were evaluated post hoc by Bonferroni's test.

FIGURE 1 Amino acid and base sequences of guinea pig cDNAs. The sequences of cDNA fragments for the collagen α1(IV) chain and laminin γ1 chain were obtained as described in Materials and Methods. Amino acid sequences were determined using the DNA Strider computer program.
Concentrations of \( \alpha_1(IV) \) collagen and elastin mRNAs in blood vessels. The sequence of the guinea pig collagen \( \alpha_1(IV) \) cDNA probe (Fig. 1) showed a high degree of homology with the human sequence (Soininen et al. 1987); 86% of bases and 92% of amino acids were identical. The guinea pig collagen \( \alpha_1(IV) \), rat elastin and 28S rRNA probes, were used for Northern blot analysis with total RNA from blood vessels, and representative blots with normal guinea pigs are shown in Figure 4. The probes hybridized with transcripts that were the expected sizes as follows: \( \alpha_1(IV) \), 5.4 and 6.7 kb (Brinker et al. 1985), and elastin, 3.6 kb (Fazio et al. 1988). The results from Northern blots with RNA from normal, scorbutic and food-deprived guinea pigs were quantitated by densitometry of autoradiograms. The values for type IV collagen and elastin mRNAs were corrected by the values for 28S rRNA, and the mean values of samples from scorbutic and food-deprived guinea pig samples were expressed as a percentage of the mean normal value (Table 3). The expression of \( \alpha_1(IV) \) collagen and elastin mRNAs was not significantly affected in scorbutic guinea pigs with moderate amounts of weight loss (0–7%). With additional weight loss, however, there were significant differences; \( \alpha_1(IV) \) collagen mRNA was 57% of normal, whereas elastin mRNA expression was 3% of normal (Table 3). In guinea pigs that were food-deprived for 3–4 d and exhibited 19.5% weight loss, the concentration of type IV

![FIGURE 4](https://example.com/figure4.png)

Representative Northern blots of collagen, elastin, and 28S rRNA transcripts in blood vessels from normal guinea pigs. Total RNA (10 \( \mu \)g) from blood vessels of two guinea pigs was electrophoresed and blotted. The blot was hybridized sequentially with cDNA probes. The sizes of transcripts in kilobases (kb) were determined from markers and are indicated at the side.
collagen mRNA was 50.5% of normal and that of elastin mRNA was 34.5% of normal (P < 0.05, Table 3).

### Western blotting for analysis of type IV collagen in serum.

Proteins containing the 7S domain of type IV collagen have been identified in human and rat sera using specifically targeted antibodies (Hasslacher et al. 1984, Hirayama et al. 1996). The concentration of immunoreactive 7S fragments has been used as a biochemical marker for assessing the synthesis of type IV collagen in humans with hepatic fibrosis (Hirayama et al. 1996) and in diabetic rats (Brocks et al. 1985, Hasslacher et al. 1984). We analyzed ammonium sulfate–precipitated serum proteins for 7S fragment on Western blots to assess type IV collagen production during vitamin C deficiency and food deprivation. To identify proteins with collagenous regions, duplicate samples were incubated without or with purified bacterial collagenase. Representative Western blots are shown in Figure 5. Reduced and denatured human placental type IV collagen specifically reacted with the antibody, displaying four chains (Fig. 5A, lane 5). These are partially degraded chains because the collagen was extracted by mild pepsin digestion, and intact chains of 185 and 170 kDa were not present. These chains were digested by purified bacterial collagenase (Fig. 5A, lane 6). Reduced and denatured serum proteins from normal and severely scorbutic guinea pigs (Fig. 5A, lanes 1–4) showed a great deal of nonspecific background compared with the type IV collagen standard. One of the nonspecific bands corresponded to the 50-kDa immunoglobulin heavy chain (IgG), which also was observed when the first antibody was omitted (Fig. 5B, lanes 3 and 4). Two proteins in normal serum were collagenous (Fig. 5A, lane 1, top two arrows) because they were digested by collagenase (Fig. 5A, lane 2), and they reacted specifically with the antibody. The major upper band corresponded to a protein of ~100 kDa, whereas the minor band was ~78 kDa, on the basis of a comparison to globular protein standards. They probably are larger, however, because collagens migrate more slowly than globular proteins of the same size. After collagenase digestion, an intensely staining band appeared at ~40 kDa (Fig. 5A, lane 2); it can be seen more clearly on a 10% gel (Fig. 5C, lane 2). Both of the collagen bands and the collagenase-resistant 40-kDa band were almost undetectable in scorbutic serum (Fig. 5A, lanes 3 and 4).

The results suggested that the 100-kDa collagen contained the 7S domain plus additional collagenous sequences and that after collagenase digestion, it gave rise to the 40-kDa fragment that consists of the basic 7S domain. The 7S domain is much more immunogenic than the triple helical region of type IV collagen (Hudson et al. 1993, Kühn 1994, Risteli et al. 1981), which would explain the intense staining of the 40-kDa fragment. The 7S domain of type IV collagen α-chains consists of a 15–20 amino acid noncollagenous sequence at the amino terminus, plus a 113–121 amino acid collagenous sequence. A monomer of this region would be ~1.6 kDa, but if it were a disulfide-bonded, lysine crosslinked trimer, it would be ~5 kDa, which suggests that the 40-kDa fragment contained additional collagenous sequences that were not accessible to collagenase. Disulfide bonds enclosing triple helical collagenous regions interfere with the action of bacterial collagenase, but reduction before digestion allows cleavage, as first documented for the N-propeptide of type III procollagen (Nowack et al. 1976). To determine if the 40-kDa 7S fragment contained such an inaccessible region, samples were reduced before collagenase treatment. The apparent 100-kDa band was still observed in the control without collagenase (Fig. 5B, lane 1), but with collagenase, it was completely digested, and the 40-kDa fragment was no longer observed (Fig. 5B, lane 2). The results suggested that the 40-kDa fragment consists of three chains, each containing the N-terminal noncollagenous domain with interchain disulphide bonds, lysine crosslinks and a short collagenous sequence containing a disulfide bond. Because the 40-kDa fragment was well separated from nonspecific bands, it was used to quantify type IV collagen on Western blots containing serum samples from two sets of normal, scorbutic and food-deprived guinea pigs. The results were quantified by densitometry, and were expressed as a percentage of the values for normal controls (Fig. 6). Because protein concentrations in sera showed little variation, results were not normalized against protein. Protein concentrations (means ± st) were as follows: normal guinea pigs (n = 6) 107 ± 8 g/L; moderate scorbutics (0–7% weight loss, n = 3) 112 ± 6 g/L; severe scorbutics (13–28% weight loss, n = 5) 115 ± 9 g/L; and food-deprived (3 and 4 d, n = 4) 103 ± 4 g/L. The concentration of the 40-kDa 7S fragment in the serum of vitamin C–deficient guinea pigs that were still growing after 16–17 d of consuming the vitamin C–free diet (0% weight loss) was in the normal range (Fig. 6A); however, with a 5–8% loss of body

### TABLE 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Col IV</th>
<th>Elastin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>mRNA2</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>3.61 ± 0.46</td>
</tr>
<tr>
<td>Scorbatic-mod</td>
<td>3</td>
<td>3.63 ± 0.33</td>
</tr>
<tr>
<td>Scorbatic-sev</td>
<td>5</td>
<td>2.06 ± 0.36</td>
</tr>
<tr>
<td>Food-deprived</td>
<td>4</td>
<td>1.83 ± 0.32</td>
</tr>
</tbody>
</table>

1 Scorbutic guinea pigs were grouped according to weight loss: moderate (mod), 0–7%; severe (sev), 13–28%. Food-deprived guinea pigs had a mean weight loss of 19.5%.

2 Northern blots containing total RNA from a single set of normal, scorbutic and food-deprived guinea pigs were analyzed as described in the legend to Figure 4. Autoradiograms were scanned twice, and the averaged values were normalized for 28S rRNA content. The results are presented as the means ± SEM of the corrected mRNA values for each group. Significance of differences between mRNA values of experimental groups and the normal guinea pigs was determined by a two-tailed Student’s t test; P-values are shown. Variances differed only for the elastin mRNA values of the severely scorbutic and food-deprived groups; therefore, they also were compared to the control group using Bonferroni’s test and were significantly different from normal guinea pigs with P < 0.001 and < 0.05, respectively.

3 Mean values of the experimental groups (E) as a percentage of normal (N) mean values.
weight, its concentration was 50–60% of normal, and with more extensive weight loss, it was 25% of normal. In serum from food-deprived guinea pigs that had lost 19–20% of their body weight (Fig. 6B), the percentages of the 40-kDa fragment were similar to those in scurbiotic guinea pigs with comparable amounts of weight loss. These observations suggested that there was a generalized down-regulation of type IV collagen production in vitamin C-deficient guinea pigs that may be related to weight loss.

DISCUSSION

This study has provided the first biochemical evidence that the expression of type IV collagen and elastin genes are down-regulated in blood vessels during vitamin C deficiency in guinea pigs. In addition, the overall metabolism of type IV collagen, as measured by the level of its 7S fragment in serum, is below normal in vitamin C deficiency. In contrast, neither expression of laminin mRNA in blood vessels nor its overall metabolism as measured by serum laminin was affected by vitamin C deficiency. Although laminin and type IV collagen interact at the structural level to form a functional basement membrane (Yurchenco and O'Rear, 1994), their regulation is not always coordinated. During the differentiation of F9 teratocarcinoma cells into parietal endoderm-like cells, mRNAs for the α1(IV) chain, and the laminin α1, β1, and γ1 chains are induced simultaneously (Kleinman et al. 1987). On the other hand, mRNAs for the type IV α-chains, and laminin β1 and γ1 chains are regulated differently in blood vessels during angiogenesis in sponge implants (Sephel et al. 1996). At a stage during angiogenesis at which mRNA levels for type IV collagen decline, laminin mRNA levels remain constant, a situation similar to that observed in blood vessels during vitamin C deficiency in this study. Our observation that the expression of elastin was reduced during vitamin C deficiency in vivo differs from the effect of ascorbate in smooth muscle cell cultures. Davidson et al. (1997) found that elastin synthesis and mRNA levels were higher in the absence of ascorbate in smooth muscle cell cultures.

The steady-state levels of mRNAs for both elastin and type IV collagen in blood vessels were lower only in scurbiotic guinea pigs with extensive weight loss (>13%), although elastin mRNA was affected more severely. A similar pattern was observed in blood vessels of food-deprived guinea pigs, in that concentrations of both elastin and type IV collagen mRNAs were lower, with elastin affected more severely.

Overall type IV collagen protein production also was lower than normal in vitamin C-deficient and food-deprived guinea pigs, based on serum levels of the 7S fragment. Several studies have provided evidence that serum concentrations of the type IV collagen 7S fragment reflect the rate of synthesis of the protein. Hasslacher et al. (1984) injected normal and diabetic rats with radioactive precursor and found that serum levels of 7S fragment correlated with changes in the rate of synthesis of type IV collagen in the basement membrane of isolated glomeruli. Brocks et al. (1985) established that such changes were not due to differences in clearance rates of the radioactive precursor. In addition, Hirayama et al. (1996), and Niemelä et al. (1985) found that 7S collagen concentrations in serum also were above normal during liver fibrosis in which new basement membranes are synthesized during capillary formation. Thus, the below normal concentration of 7S fragment in scurbiotic guinea pig serum may reflect an overall down-regulation of type IV collagen production in the vascular system, as well as in other tissues. Because the concentration of immunoreactive laminin fragments in serum closely reflects laminin mRNA concentrations in blood vessels, it also seems likely that they are derived at least in part from blood vessels. The immunoreactive laminin and type IV collagen proteins were considerably smaller than intact chains, suggesting that they were derived by degradation of newly synthesized molecules, as previously suggested (Brocks et al. 1985).

The similar effects of vitamin C deficiency and food depri-
levels, which in turn increases IGFBP-1 and -2 expression in the liver and leads to higher levels of circulating IGFBP-1 and -2 (Gosiewska et al. 1994) that are taken up into tissues (Gosiewska and Peterkofsky 1995). IGFBP-1 and -2 inhibit the action of IGF-1 and thus can cause inhibition of DNA synthesis, collagen gene expression in bone, skin and cartilage (Gowiewska et al. 1994, Peterkofsky et al. 1994), and cartilage proteoglycan synthesis (Bird et al. 1986).

The down-regulation of type IV collagen in vitamin C–deficient guinea pigs most likely occurs through the same mechanism as for types I and II collagens because IGF-1 plays a role in the regulation of several types of collagen. IGF-1 stimulates the expression of the genes for type I collagen in fibroblasts and type II collagen in chondrocytes (Goldstein et al. 1989, Peterkofsky 1991). In growth hormone–deficient dwarf rats, administration of growth hormone, whose actions are mediated mainly through IGF-I, induces mRNAs for IGF-I and types I and III collagens (Wilson et al. 1995). More relevant to this study are reports that administration of growth hormone to rats increases collagen deposition in the aortic intima (Bruel and Oxlund 1995), and that IGF-1 stimulates type IV collagen mRNA expression and production of the protein in cultured mesangial cells (Feld et al. 1995). Transcription of the elastin gene in smooth muscle cells is stimulated also by IGF-1 (Conn et al. 1996). Both vascular smooth muscle and endothelial cells in culture express IGF-I (Delafontaine et al. 1991), and immunoreactive IGF-I and its mRNA are found in the smooth muscle layer of aorta (Murphy et al. 1990). Thus, type IV collagen and elastin genes in these cells could be regulated by IGF-I through endocrine and autocrine mechanisms, and inhibited by IGFBP-1 and -2 that is taken up into tissues from the circulation (Gosiewska and Peterkofsky 1995).

In summary, there is lower expression of type IV collagen and elastin, but not laminin, in blood vessels of vitamin C–deficient guinea pigs. Lower concentrations of serum type IV collagen suggest that overall production of the protein also is down-regulated during vitamin C deficiency, possibly in a number of tissues as well as in the vascular system. These effects on elastin and type IV collagen metabolism in vitamin C–deficient guinea pigs are most likely mediated through the induction of circulating IGFBP-1 and -2 after weight loss has commenced. Because of the roles of type IV collagen and elastin in maintaining the structural integrity of blood vessel extracellular matrices, reduction in the levels of these proteins could contribute to structural defects in blood vessels that were observed previously in vitamin C–deficient guinea pigs.

**LITERATURE CITED**


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