Topically Applied Vitamin C Enhances the mRNA Level of Collagens I and III, Their Processing Enzymes and Tissue Inhibitor of Matrix Metalloproteinase 1 in the Human Dermis[1]

Betty V. Nusgens, Philippe Humbert,* André Rougier,† Alain C. Colige, Marek Haftek,‡ Charles A. Lambert, Alain Richard,† Pierre Creidi,* and Charles M. Lapière

Laboratory of Connective Tissues Biology, Tour de Pathologie, University of Liège, Sart Tilman, Belgium; *Department of Dermatology, Hôpital Saint Jacques, University of Franche-Comté, Besançon, France; †La Roche-Posay, Laboratoire Pharmaceutique, Asnières, France; ‡INSERM U346/CNRS, Hôpital E. Herriot, Lyon, France

Ascorbic acid (vitamin C) is a cofactor required for the function of several hydroxylases and monooxygenases. It is not synthesized in humans and some other animal species and has to be provided by diet or pharmacologic means. Its absence is responsible for scurvy, a condition related in its initial phases to a defective synthesis of collagen by the reduced function of prolylhydroxylase and production of collagen polypeptides lacking hydroxyproline, therefore, they are unable to assemble into stable triple-helical collagen molecules. In fibroblast cultures, vitamin C also stimulates collagen production by increasing the steady-state level of mRNA of collagen types I and III through enhanced transcription and prolonged half-life of the transcripts. The aim of the experimental work has been to evaluate the effect on dermal cells of a preparation of vitamin C topically applied on one side *vs* placebo on the other side of the dorsal face of the upper forearm of postmenopausal women. Biopsies were collected on both sides and the level of mRNA measured by non competitive reverse transcription–polymerase chain reaction made quantitative by the simultaneous transcription and amplification of synthetic RNA used as internal standards. The mRNA of collagen type I and type III were increased to a similar extent by vitamin C and that of three post-translational enzymes, the carboxy- and amino-procollagen proteinases and lysyloxidase similarly increased. The mRNA of decorin was also stimulated, but elastin, and fibrillin 1 and 2 were not modified by the vitamin. The expression of matrix metalloproteinases 1, 2, and 9 was not significantly changed, but an increased level of tissue inhibitor of matrix metalloproteinase 1 mRNA was observed without modification of tissue inhibitor of matrix metalloproteinase 2 mRNA. The stimulating activity of topical vitamin C was most conspicuous in the women with the lowest dietary intake of the vitamin and unrelated to the level of actinic damage. The results indicate that the functional activity of the dermal cells is not maximal in postmenopausal women and can be increased. ***Key words: ADAMTS2/BMP1/decorin/elastin/matrix metalloproteinases. J Invest Dermatol 116:853–859, 2001***

L-ascorbic acid (vitamin C) is an essential nutrient for some animal species and humans that lack the last enzyme in the pathway for its synthesis from glucose (Banhegyi *et al*, 1997). Vitamin C is required for the optimal functioning of several hydroxylases and monooxygenases (Padh, 1990). Its absence is responsible for scurvy, a disease characterized by altered functions of the connective tissues, including perifollicular hemorrhages and defective healing. Scurvy was a most usual cause of morbidity and mortality in sailors during the fifteenth century and up to the end of the eighteenth century in the British Navy (Thomas, 1997). It was largely prevented by the introduction of lemon juice in the diet. At the present time, scurvy still exists in the Western world (Hirschmann and Raugi, 1999), mainly in institutionalized patients, drug addicts, and alcoholics that consume food deprived of vitamin C. The minimal dietary intake of vitamin C to prevent scurvy is 6.5 mg per day (Bartley *et al*, 1953) and is barely large enough to prevent a drop of the body pool of the vitamin below 50%. The recommended daily intake to keep a saturated pool of the vitamin is much larger and about 80 mg (Levine *et al*, 1999) contained in five servings of vegetables (fresh or barely cooked) or fruits per day. In the absence of chemical supplementation saturation is rarely achieved.

It is presently known that vitamin C is required as a cofactor for the correct hydroxylation of prolyl and lysyl residues of the procollagen polypeptides (Kivirikko and Prockop, 1967) allowing their triple helical conformation in the cells (Rosenbloom *et al*, 1973) and the secretion, processing, and polymerization of these

Manuscript received May 26, 2000; revised February 23, 2001; accepted for publication February 26, 2001.

Reprint requests to: Dr. Betty V. Nusgens, Laboratory of Connective Tissues Biology, Tour de Pathologie, University of Liège, B23/3, 4000 Sart Tilman, Belgium. Email: LCTB@ulg.ac.be

Abbreviations: 28S rRNA, ribosomal RNA; sRNA, synthetic RNA; α_1 I, collagen I α_1 chain; α_1 III, collagen III α_1 chain; N-PCP, amino-procollagen proteinase (ADAMTS2); C-PCP, carboxy-procollagen proteinase (BMP1); LO, lysyloxidase; MMP-1, matrix metalloproteinase 1; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; TIMP, tissue inhibitors of matrix metalloproteinases.

[1]Part of this work was presented in poster form at the American Academy of Dermatology, San Francisco, CA, March 10–15, 2000.

precursors to form the fibers ultimately conferring resistance to the tissues (Levene, 1975). In fibroblast cultures, vitamin C stimulates collagen biosynthesis (Peterkofsky, 1972) not only by promoting the activity of the hydroxylases, but also by increasing the steady-state level of the procollagen mRNA (Lyons and Schwarz, 1984; Schwarz, 1985; Pinnell *et al*, 1987). This depends on both an increased transcription of the genes and a stabilization of the transcripts to a similar extent for the mRNA of the main types of collagen (I and III) present in skin (Geesin *et al*, 1988).

The effect of vitamin C on the level of elastin mRNA in cultured fibroblasts is the reverse. It reduces transcription and decreases the stability of the transcripts (Davidson *et al*, 1997).

Besides its cofactor activity, vitamin C is also a free radical scavenger by its antioxidant properties (for a review see Sauberlich, 1994). It protects keratinocytes from the damage produced by ultraviolet A (Tebbe *et al*, 1997). Its beneficial activity as a photoprotectant (Darr *et al*, 1996) and anticancer agent (Pauling, 1991) has been demonstrated by dietary supplementation in humans and in animal species even in those that can synthesize the vitamin. A photoprotective effect has also been demonstrated after topical application (Darr *et al*, 1992; Colven and Pinnell, 1996). Vitamin C is also required to form competent barrier lipids in the epidermis (Ponec *et al*, 1997) by stimulating the synthesis of ceramides. It has also been shown to stimulate the barrier function of the endothelial cells (Utoguchi *et al*, 1995).

The aim of this study was to evaluate the effect on the dermal cells of vitamin C administrated by topical application on the skin of normal human volunteers by measuring: the steady-state level of the mRNA of procollagen I and III, their post-translational processing enzymes, carboxy-procollagen proteinase (Li *et al*, 1996), amino-procollagen proteinase (Colige *et al*, 1997) and lysyloxidase (LO) (Hamalainen *et al*, 1991), decorin, a collagen fiber-associated proteoglycan (Danielson *et al*, 1997), the main components of the elastic fibers, elastin and fibrillins 1 and 2 (Ramirez and Pereira, 1999) as well as the metalloproteinases and their physiologic inhibitors involved in the degradation of these matrix components (Nagase and Woessner, 1999). Such investigations performed on small biopsies were made possible by the use of quantitative reverse transcription–polymerase chain reaction (RT–PCR) controlled by original newly created internal standards of synthetic RNA.

MATERIALS AND METHODS

Selection and treatment of the volunteers Ten human volunteers were selected from a cohort of postmenopausal women 50–60 y old (mean 55.3 y). Most testers included in the study received a substitutive hormonal therapy, except tester no. 10 who received only progesterone. No vitamin supplementation was allowed during the test. The study was conducted between December and June and the testers were instructed to avoid sun exposure. No other topically applied preparation was permitted. The treatment consisted of applying, at night, on the dorsal side of the upper forearm preparation A (active) containing 5% vitamin C on one side and preparation P (placebo) on the other side. The distribution of the preparations, active and placebo, was randomly allocated and not known by the testers or by the investigators. The once-daily application was repeated for 6 mo, the tester being examined after 3 and 6 mo for clinical observation and detection of potential side-effects. The tolerance was perfect. At the termination of treatment, two 5 mm punch biopsies up to the hypodermis were collected under local anesthesia at the site of the topical application. One biopsy was used for measurement of mRNA and collagen extractability. The second biopsy was used for classical morphology and electron microscopy to evaluate sun damage. The study was conducted in agreement with the Declaration of Helsinki and approved by the ethical committee of the University Hospital Saint-Jacques in Besançon, France.

Preparation of the stabilized w/o emulsion containing 5% vitamin C The active preparation (A) was a solution of 5% L-ascorbic acid (vitamin C) in glycerol at pH 6.0 emulsified in a silicone base and prepared under an atmosphere of nitrogen, commercially available (Active C, La Roche-Posay, France). The placebo (P) was the same preparation devoid of vitamin C. The preparations were kept in similar aluminum tubes preventing contact with air. The stability of the preparations was longer than 3 y at room temperature.

Extraction and purification of the RNA Immediately after sampling, one biopsy from each side was freed of adhering hypodermis, wrapped in aluminum foils, placed in a small vials dropped and kept in liquid nitrogen until use. Half of the biopsy was ground in liquid nitrogen in a Microdismembrator S (Braun Biotech International, Melsungen, Germany) and the resulting powder collected in 2 ml of the lysis solution (5 M guanidium isothiocyanate, 0.1 M β-mercaptoethanol, 17 mM Na laurylsarcosyl, 25 mM Na citrate pH 7.0). After 15 min of agitation at room temperature the lysate was overlaid on a cushion of 1.4 ml 5.7 M cesium chloride, 0.1 M ethylenediamine tetraacetic acid pH 7.0 and ultracentrifuged (110,000 × *g*, 18 h at 20°C) in a SW 60 rotor (Beckman L70M, Palo-Alto, CA). The pellet of RNA was rinsed in 70% ethanol, centrifuged at 6800 × *g* (4°C, 10 min), and dissolved in 500 μl RNAse-free distilled water. The concentration of RNA and its purity were estimated by optical density at 260 nm and 260/280 nm ratio. The stock solutions of RNA were diluted to a concentration of 4 ng per μl, aliquoted, and stored at –80°C.

Reverse transcription–PCR assay The specific oligonucleotide primers of the mRNA of interest were around 24 bases long with an A-T proportion close to 50%. The sequences shown in **Table I** were chosen on different exons to allow discrimination of products amplified from potential contaminating DNA. The mRNA of interest and the 28S rRNA were quantified in triplicate by reverse transcription–PCR using 10 ng of total RNA. The RNA were reverse transcribed at 70°C for 15 min and amplified (94°C for 15 s, 66°C for 20 s, 78°C for 10 s) using the Gene Amp rTth Kit (Perkin Elmer, Branchburg, NJ), except matrix metalloproteinase (MMP) -2 that was reverse-transcribed at 55°C for 30 min and amplified (94°C for 18 s, 62°C for 20 s, 68°C for 15 s) using the kit Titan (Boehringer, Mannheim, Germany). The number of amplification cycles used in this study is indicated in **Table I**. The efficiency of the reverse transcription and the amplification reactions was monitored by adding in each tube an appropriate number of copies of a synthetic RNA (sRNA) that can be reverse-transcribed and amplified with the primers pair used for the amplification of the mRNA of interest, but giving rise to a product slightly larger or smaller than the product amplified from the mRNA (**Table I**), allowing its discrimination after migration in a 10% polyacrylamide gel. The different sRNA were generated from linearized template plasmid containing appropriate DNA inserts by the use of SP6 RNA polymerase (SP6/T7 transcription kit; Boehringer Mannheim), purified (High Pure RNA Isolation kit; Boehringer Mannheim) and quantitated[2] (manuscript in preparation). The gels were stained with SyberGreen (Biorad, Hercule, CA) or Gelstar (FMC Bioproducts, Rockland, ME) and the intensity of the fluorescent signals measured in a Fluor-S-MultiImager (Biorad).

For each of the mRNA, the optimal conditions of reaction (temperature, number of cycles and choice of the reverse transcription–PCR kit) and the amount of internal standard were determined taking into account the level of expression of the mRNA in human skin to obtain values within the linear range of measurement as recommended by Freeman *et al* (1999) and illustrated in **Fig 1**. Each measurement was normalized to the cotranscribed and coamplified internal standard. The normalized values were expressed in arbitrary units (AU) per unit of 28S rRNA measured in the same dilution of RNA to correct for RNA input of each sample.

Measurement of collagen extractability The remaining half of the biopsy was used to measure collagen after sequential fractionation. The fragments of skin were lyophilized, weighed, and extracted at 4°C in 0.5 M acetic acid-HCl (HAc) at pH 2.0 for 24 h followed by extraction after pepsin digestion (200 μg per ml) in 0.5 M acetic acid-HCl pH 2.0 for a further 24 h. Aliquots of the extracts and the residual material were hydrolyzed in 6 M HCl and the concentration of hydroxyproline was determined by a colorimetric assay (Bergman and Loxley, 1963).

Morphologic analysis The second biopsy from the placebo-treated side was separated in two fragments perpendicularly to the stratum corneum. One fragment was fixed in Baker's solution for 3 h and embedded in paraffin. The other fragment was further sectioned in smaller samples and fixed in 2% glutaraldehyde in cacodylate buffer for 6 h, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and

[2]Lambert ChA, Colige AC, Maniglia S, Heyeres A, Munaut C, Lapière ChM. and Nusgens BV: Measurement of matrix metalloproteinases by quantitative RT-PCR assay: Application to the study of gene regulation in fibroblasts by stress relaxation. *J Invest Dermatol* 110:612, 1998 (abstr.)

Table I. Nucleotide sequence of the primers, size of the generated reverse transcription–PCR products and number of cycles for amplification

RNA[a]	Primers		Size of the products (bp)		Cycle number
	Forward (5′-3′)	Reverse (5′-3′)	Endogenous	sRNA	
28S rRNA	GTTCACCCACTAATAGGGAACGTGA	GGATTCTGACTTAGAGGCGTTCAGT	212	269	18
GAPDH	CCTGGCCAAGGTCATCCATGACA	GGGATGACCTTGCCCACAGCCTT	183	262	25
Keratin 10	GGGAGCCTCGTGACTACAGCAAAT	CCCTACGCAGGCCGTTGATGTCA	201	None	28
Vimentin	GACAATGCGTCTCTGGCACGTCTT	TCCTCCGCCTCCTGCAGGTTCTT	230	None	30
α1(I)	CCCACCAATCACCTGCGTACAGA	TTCTTGGTCGGTGGGTGACTCTGA	214	267	27
α1(III)	GAGATGTCTGGAAGCCAGAACCAT	GATCTCCCTTGGGGCCTTGAGGT	207	265	27
N-PCP	GAACCATGAGGACGGCTTCTCCT	GGCTGCAGCGGGACCAGTGGAA	176	261	35
C-PCP	AAGTCCGACAACACCGTGTCCAAA	CCATCCCATTCCGTCCAAGGTGA	201	265	35
LO	CCCCTACAAGTACTCTGACGACAA	CGCCGCGCATCTCAGGTTGTACAT	202	263	30
Decorin	CCTGAAAGGACTGAATAATTTGGCTA	GTTGCTGAAAAGACTCACACCCGAA	277	201	30
Elastin	CCGCTAAGGCAGCCAAGTATGGA	AGCTCCAACCCCGTAAGTAGGAAT	275	197	28
Fibrillin 1	GGTGAATGTACAAACACAGTCAGCA	ATAGGAACAGAGCACAGCTTGTTGA	275	210	30
Fibrillin 2	ATGGCTCTCGATGCATCGATCAGA	CATTGCCACTTGGGGCAAAGCCA	282	199	35
MMP-1	GAGCAAACACATCTGAGGTACAGGA	TTGTCCCGATGATCTCCCCTGACA	185	267	35
MMP-2	AGATCTTCTTCTTCAAGGACCGGTT	GGCTGGTCAGTGGCTTGGGGTA	225	271	27
MMP-9	GCGGAGATTGGGAACCAGCTGTA	GACGCGCCTGTGTACACCCACA	208	266	34
TIMP1	CATCCTGTTGTTGCTGTGGCTTGAT	GTCATCTTGATCTCATAACGCTGG	170	270	30
TIMP2	CTCGCTGGACGTTGGAGGAAAGAA	AGCCCATCTGGTACCTGTGGTTCA	155	269	25

[a]The identity of the investigated RNA is described in the text.

embedded in Epon. Ultrathin sections were counterstained with lead citrate and uranyl acetate. Five micrometer paraffin sections were stained with hematoxylin–eosin–saphran, orcein, or immunolabeled using monoclonal antibodies to vimentin (VIM 3B4, Progen, Heidelberg, Germany) and collagen IV (clone CIV22, Dako, Glostrup, Denmark), both revealed with biotin-streptavidin-alkaline phosphatase (LSAB kit, Dako). Light and electron microscopy were used to evaluate sun damage by comparison with nonexposed buttock skin of five age-matched individuals. Three parameters were analyzed: irregularity of the epidermal pigmentation and disorganization of the dendritic cells network, flattening of the epidermal–dermal junction and subepidermal hyaline bodies, and disorganization of the elastic fiber network. Each parameter was scored semiquantitatively (0, no alteration; 1, very light; 2, moderate; and 3, severe) and summed to determine the extent of solar alteration.

Statistical analysis Results were expressed as the ratio between the values measured in the skin sample treated with the preparation containing vitamin C (A) and that treated with the placebo (P). The mean ratio was tested for statistical difference from 1 by using the one-sided Student's t test for paired data.

RESULTS

The biopsies contain similar proportions of dermal and epidermal RNA The contribution of the dermis in the collected RNA was estimated by measuring the steady-state level of vimentin (VIM) mRNA, the protein of the intermediate filaments of the mesenchymal cells whereas that of the epidermis was obtained by measuring the mRNA of keratin 10 (K10) (**Table II**). The mean ratio VIM/K10 was similar on both sides, that treated with vitamin C (A) and the controlateral side receiving the placebo (P), indicating a similar contribution of epidermal and dermal RNA in the samples; however, the values of VIM and K10 per unit of 28S rRNA were both increased in the vitamin C-treated side, which resulted in an A/P ratio significantly higher than 1.0. The mean value for the mRNA level of the housekeeping gene GAPDH per unit of 28S rRNA was very similar on both sides resulting in an A/P ration close to 1.0 (**Table II**).

The studied population is heterogeneous in terms of level of expression of all the investigated genes. This is clearly illustrated in **Fig 2** for the procollagen processing enzyme, the C-PCP ranged in the studied population from 65 to 370 AU per unit of 28S rRNA. The individual variations were, however, similar on both arms resulting in a mean value almost identical for the group of samples collected on the right (R) and the left (L) arm and an R/L ratio close to 1.0. To account for that heterogeneity the results in the tables were mostly expressed by the ratio of the values observed in the vitamin C-treated side (active: A) to that of the placebo (P). In the example illustrated in **Fig 2**, the A/P ratios significantly higher than 1.0 indicate an increased steady-state level of C-PCP mRNA upon vitamin C treatment.

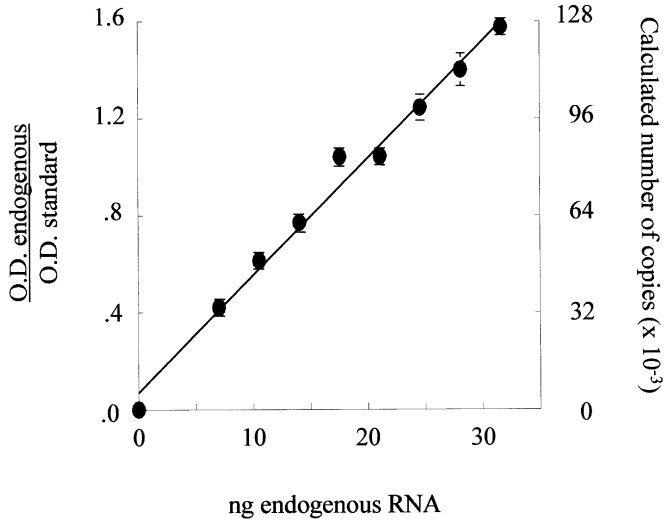

Figure 1. Titration of the mRNA of collagen α1 (I). The ratio of the signals obtained for the amplification products of increasing amounts of endogenous RNA with the addition of a constant amount (85,000 copies) of standard synthetic RNA increases linearly (r = 0.998) with the amount of endogenous RNA. Each value is the average of triplicate measurements ± SD.

The mRNA levels of collagen type I and type III and that of three extracellular procollagen processing enzymes are coordinately increased by vitamin C The steady-state level of the mRNA of the two main types of collagen in skin, types I and III, was measured by quantitative reverse transcription–PCR using specific sRNA as internal standards for the α1 chains of collagen I

Table II. The RNA collected from the biopsies arises in a constant proportion from the epidermis (K10) and the dermis (VIM)[a]

Tester	VIM/K10 A	VIM/K10 P	K10 A/P	VIM A/P	GAPDH A/P
1	7.22	6.05	1.58	1.88	1.76
2	6.88	[14.10] [b]	[2.64][b]	1.29	0.69
3	5.27	5.63	0.93	0.87	0.82
4	4.53	2.68	0.90	1.53	1.16
5	4.82	4.18	1.17	1.35	1.51
6	2.65	4.31	1.68	1.03	0.87
7	3.59	6.34	1.88	1.06	1.35
8	3.77	5.36	1.17	0.83	0.62
9	3.21	4.48	1.23	0.88	0.64
10	4.19	4.93	1.46	1.24	1.14
Mean	4.61	4.88	1.33	1.20	1.06
± SD	± 1.50	± 1.12	± 0.34	± 0.33	± 0.39
	NS[c]	NS	$p < 0.01$	$p < 0.05$	NS

[a]K10 and VIM mRNA levels are significantly increased in vitamin C treated sides (A) as compared with placebo (P), whereas GAPDH mRNA level is not modified by the vitamin.
[b]Ectopic values excluded from mean and SD.
[c]NS, not significant.

Table III. The topical application of vitamin C (A) increases the steady-state level of the mRNA of collagen I (α1 I) and collagen III (α1 III) in the same six of the 10 testers without disturbing the ratio between the two types of collagen (I/III)

Tester	α1 I A/P	α1 III A/P	I/III A	I/III P
1	1.60	1.34	2.91	2.45
2	1.38	1.64	2.58	3.07
3	0.92	0.91	2.84	2.75
4	1.55	1.56	3.01	3.02
5	1.74	1.59	2.72	2.49
6	0.89	0.96	2.59	2.80
7	1.24	1.24	2.55	2.56
8	0.71	0.62	3.02	2.64
9	0.83	0.78	2.76	2.57
10	1.62	1.45	3.04	2.72
Mean	1.25	1.21	2.80	2.71
± SD	± 0.38	± 0.37	± 0.19	± 0.21
	$p < 0.04$	$p < 0.06$	NS[a]	NS

[a]NS, not significant.

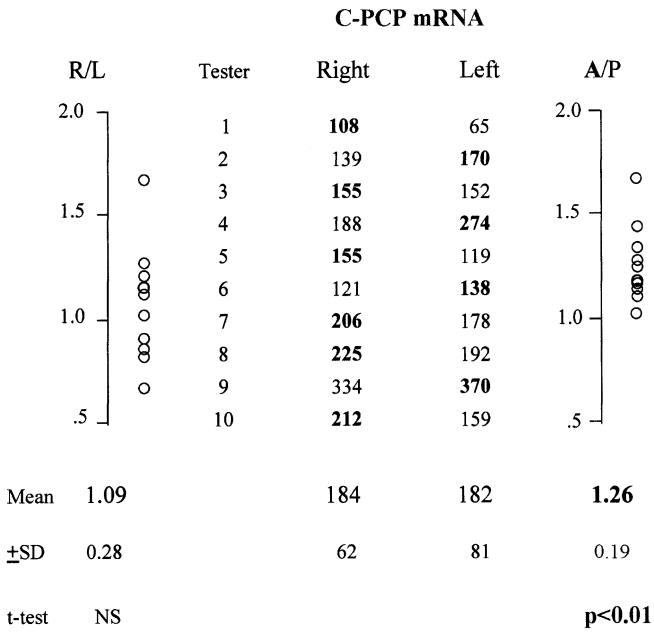

Figure 2. Heterogeneity of the tested population. The mRNA level (in arbitrary units per unit of 28S sRNA) for C-PCP varies among the testers but similarly on the right (R) and the left (L) sides resulting in a mean R/L ratio close to 1.0 (left part). The stimulating effect of topical vitamin C (in bold figures) is expressed by a mean ratio active/placebo (A/P) significantly higher than 1.0 (right part).

Table IV. The mRNA steady-state level of the processing (N-PCP and C-PCP) and cross-linking (LO) enzymes is increased on the side treated by vitamin C (A) by comparison with the side treated with placebo (P)

Tester	N-PCP A/P	C-PCP A/P	LO A/P
1	1.33	1.66	5.67
2	1.10	1.22	2.53
3	0.87	1.02	0.57
4	1.96	1.46	1.18
5	1.58	1.30	2.20
6	1.19	1.14	1.41
7	1.00	1.16	2.55
8	0.86	1.17	0.24
9	0.79	1.11	1.47
10	1.41	1.33	1.45
Mean	1.21	1.26	1.93
± SD	± 0.37	± 0.19	± 1.52
	$p < 0.06$	$p < 0.01$	$p < 0.05$

and III. The example of titration of the collagen polypeptides α1 (I) mRNA (**Fig 1**) illustrates the linearity, reproducibility, and sensitivity of the procedure.

The variability in the level of the two collagen mRNA was lower than that observed for C-PCP and similar on both sides (not illustrated). Six of the 10 testers displayed increased values for both types of collagen on the side treated with the active preparation and the mean A/P ratio of the whole group for both collagen type I and type III supported a stimulating effect of the vitamin (**Table III**). This occurred to the same extent with the two types of collagen. The ratio between collagen I and collagen III was very similar on both sides in spite of the individual variability in the level of these mRNA within the group of testers.

The interindividual variability of LO was higher than that of C-PCP and N-PCP. The C-PCP mRNA enhancing activity of vitamin C is illustrated in **Fig 2** by a statistically significant increase of the mean A/P ratio. The mean values of the A/P ratios were also significantly increased for the two other enzymes (N-PCP and LO) involved in the maturation of the (pro)collagen molecules (**Table IV**).

The concentration of collagen and its extractability are not significantly modified in the vitamin C treated skin The fraction of acetic acid-extracted collagen was very small in agreement with previously published data (Legrand *et al*, 1969). The proportion of extracted collagen and that remaining in the insoluble residue was similar in the two groups of samples and the mean A/P ratio did not significantly differ from 1 (**Table V**).

The mRNA of decorin and TIMP1 are increased by vitamin C but not that of elastin, fibrillins, MMP-1, MMP-2, MMP-9, and tissue inhibitor of matrix metalloproteinase (TIMP) 2 The mean value of the mRNA for elastin and fibrillin 1 collected from the vitamin C-treated was not significantly increased as compared with placebo (**Table VI**). The mRNA of decorin was increased by the topical application of vitamin C. The difference was, however, at the limit of significance. The transcript of fibrillin 2 was not reproducibly detected.

The expression of MMP-1 was very small, barely detectable in most instances, whereas a few samples displayed higher values. There was no statistically valid difference between the two sides. The interindividual expression of MMP-2 was also variable and no significant difference was observed between the side treated with the vitamin and the placebo. The same conclusions apply to MMP-9 (**Table VI**); however, the mRNA level of TIMP1 was increased on the side treated with the active preparation (**Table VI**), but not that of TIMP2.

Table V. Extractability of collagen in vitamin C-treated (A) and placebo-treated (P) skin

	A (n = 10)	P (n = 10)	A/P (n = 10)	t test
0.5 M HAc	8.3 ± 3.3[a]	8.7 ± 3.7	1.30 ± 1.20	NS
Pepsin	55 ± 16	58 ± 16	0.98 ± 0.27	NS
Insoluble	373 ± 103	349 ± 60	1.07 ± 0.23	NS
Total	436 ± 110	416 ± 64	1.05 ± 0.21	NS

[a]In μg of collagen per mg of tissue, dry weight.

Table VI. Expression of a series of other connective tissue related genes upon topical application of vitamin C

	A/P[a]	± SD	t test
Elastin	1.10	± 0.63	NS[b]
Fibrillin 1	1.24	± 0.69	NS
Decorin	1.64	± 1.27	$p < 0.10$
MMP1	4.50	± 6.30	NS
MMP2	1.51	± 1.09	NS
MMP9	1.41	± 0.94	NS
TIMP1	1.38	± 0.54	$p < 0.03$
TIMP2	1.27	± 0.84	NS

[a]Mean value of A/P ratios.
[b]NS, not significant.

Relationship between the metabolic effect of topical vitamin C, the dietary intake of vitamin C and the extent of actinic damage The dietary intake of vitamin C has been estimated after termination of the study by a questionnaire collecting semi-quantitative information to estimate the consumption of fruits and vegetables. It is expressed as the sum of servings per day. Smoking was also recorded as it increases the need for a higher intake of vitamin C to contribute to the pool. This information has been gathered in **Table VII** and correlated with the potential of topical vitamin C to modulate the biosynthetic activity of the cells. It is worth noting that the testers that have the lowest score of dietary vitamin C intake are those in which topically applied vitamin C displayed the most constant stimulation of the steady-state level of mRNA for collagens and their processing enzymes.

All 10 subjects presented histologic and ultrastructural signs of actinic damage (**Table VII**). There was, however, no correlation between its extent (from 2, very mild, to 8, extensive) and the responsiveness to the topically applied vitamin C.

DISCUSSION

The presented data demonstrate that daily topical application of a preparation containing 5% L-ascorbic acid enhances the steady-state level of procollagens I and III mRNA and that of their post-translational maturation enzymes. The mRNA level of the MMP responsible for the degradation of the extracellular matrix is not statistically modified, whereas the mRNA of TIMP1, a physiologic inhibitor of MMP, is increased. These modifications reflect the expression of an anabolic phenotype. This pattern of enhanced anabolic activity has been obtained by performing the mRNA measurements in a small cohort of only 10 female volunteers at the site of application of the active preparation compared with the excipient alone on the opposite parallel site. This strategy allowed us to establish a ratio between paired values in the same individual, therefore, minimizing the bearing of a marked interindividual heterogeneity in the expression of most mRNA on the statistical significance of the results.

The reverse transcription–PCR procedure used for measuring the biosynthetic activity of the cells in small samples of tissues has been carefully controlled to generate comparative data with a high degree of confidence. Our quantification procedure of ribosomal (28S rRNA) and specific mRNA by reverse transcription–PCR under noncompetitive conditions used an internal standard of sRNA and is most adequate to demonstrate small differences (Freeman *et al*, 1999). We have verified that in each biopsy the RNA arises in a similar proportion from the epidermis by measuring the mRNA of K10 and from the dermis by measuring the mRNA of VIM. This condition has to be fulfilled to permit a reliable estimation of the mRNA of interest on the basis of a unit

Table VII. The testers with the lowest intake of vitamin C are most responsive to topically applied ascorbic acid

Dietary vitamin C[a]	Smoking[b]	Testers	α1 I	α1 III	N–PCP	C–PCP	LO	Decorin	Elastin	Fibrillin 1	MMP2	MMP9	TIMP1	TIMP2	Actinic damage[c]
2	ND	10	+[d]	+	+	+	+	+	+	+	–	+	+	+	5
3	0	5	+	+	+	+	+	+	+	+	+	+	+	+	5
4	10	4	+	+	+	+	+	+	+	+	–	–	+	+	6
4	3	2	+	+	+	+	+	+	–	–	+	–	+	+	6
4	0	1	+	+	+	+	+	+	+	+	+	–	+	+	7
4	0	7	+	+	=	+	+	+	+	+	+	+	+	+	3
5	0	6	–	–	+	+	+	+	+	+	+	+	+	+	2
5	0	9	–	–	–	+	+	=	–	–	–	+	–	–	7
5	0	8	–	–	–	+	–	–	–	–	–	–	–	–	8
6	0	3	–	–	–	+	–	–	–	–	–	–	+	–	7

[a]Semiquantitative estimation of the dietary intake of vitamin C expressed as the sum of servings of fruits and vegetables per day.
[b]Cigarettes per day.
[c]Semiquantitative estimation (see *Materials and Methods*).
[d]+, A/P ratio > 1; –, A/P ratio < 1; =, A/P ratio = 1; ND, not determined.

amount of 28S rRNA. The validity of the procedure is supported by the constant ratio of collagen type I and type III mRNA level in the biopsies, similar to the ratio of these collagens in adult human skin (Epstein, 1974) and that produced by human fibroblasts in culture (Phillips *et al*, 1992).

In fibroblast cultures, the mRNA of the two main types of collagens are known to be coordinately upregulated by vitamin C (Geesin *et al*, 1988). This also seems to be the case in our *in vivo* study. Furthermore, the mRNA level of the three enzymes involved in the post-translational processing and cross-linking of the collagen molecules was also stimulated by the vitamin C treatment suggesting the existence of a mechanism of regulation parallel to that operating for the collagen polypeptides. An increased activity of the N-PCP is known to exist in pathologic conditions where the biosynthetic activity of the connective tissues is enhanced (Lapière and Piérard, 1974). The repercussion at the tissue level of the increased expression of procollagens mRNA in vitamin C treated skin was tentatively evaluated by measuring the amount of extractable collagen at acid pH (Legrand *et al*, 1969) and after pepsin digestion as an indirect indicator of collagen turnover rate, the newly synthetized collagen being less cross-linked and more extractable (Gross, 1959). In the adult human, the half-life of dermal collagen might be as long as 10 y by extrapolation of animal data (Nimni and Bavetta, 1964; Nimni M., personal communication) and the amount of replaced collagen about 10 μg per mg dry skin during the period of treatment by the vitamin. The 30% increase induced by vitamin C (3 μg in mass of which only a small fraction can be extracted) is too small to be detected. The parameters estimating the level of cell activity are obviously more sensitive.

The daily requirement of dietary vitamin C to prevent scurvy, i.e., to permit at least the vital function of the collagen hydroxylases and other monooxygenases, has been estimated at less than 10 mg per day (i.e., 15 ml of lemon juice). Such a low intake is compensated by a sparing mechanism of cellular recycling of the oxidized vitamin by two enzyme systems (Banhegyi *et al*, 1997). A daily intake of 60–100 mg vitamin C is required to raise the serum level in humans to that of animals that synthesize L-ascorbate. This amount needs to be increased to 140 mg per day for smokers that seem to consume vitamin C to inactivate oxidants from tobacco smoke (Schectman, 1993). The concentration of ascorbate is greater in the skin than that in plasma, probably by a mechanism of active transport (Welch *et al*, 1995). Nevertheless, our data demonstrate that in postmenopausal women the topical application of vitamin C is able to produce a coordinated increase in the steady-state level of the mRNA for collagen I and III as observed in six testers, and of their post-translational extracellular enzymes, as observed in at least, seven of the 10 testers. We speculated that the nonresponders might already have a concentration of the vitamin in skin great enough for maximal expression of the ascorbate-responsive mRNA. This hypothesis is supported by the ranking of the testers (**Table VII**) showing an inverse relationship between the responsiveness to vitamin C and the overall dietary intake of the vitamin. An indirect effect of vitamin C mediated by the epidermis, however, cannot be ruled out as the epithelial cells were also stimulated by the topical application of the vitamin. Keratinocytes are known to produce *in vitro* several cytokines that are modulated by vitamin C (Tebbe *et al*, 1997).

The dermal signs of aging, photoinduced and chronologic, are to some extent comparable with scurvy, i.e., atrophy, fragility, easy bruising, and palor. In aging, the density of the dermal collagen network diminishes by reduced metabolic activity (Legrand *et al*, 1969), and enhanced degradation by increased production of MMP (Millis *et al*, 1992), perhaps related to an oxidative stress (Sohal and Weindruch, 1996) similar to that induced by ultraviolet in fibroblasts (Wlaschek *et al*, 1995). The level of three MMPs involved in acute (Fischer *et al*, 1999) or chronic (Seite *et al*, 2000) skin alterations induced by ultraviolet irradiation was not significantly modified by the topical application of the vitamin. The activity of these proteases is known to be reduced *in vivo* by vitamin A (Varani *et al*, 2000) through a mechanism of action probably different from that operating for the vitamin C. The expression of TIMP1, but not TIMP2, however, was increased suggesting that the MMP activity in the vitamin C-treated side may be balanced by their physiologic inhibitor. Chronic photoaging also features an increased deposition of abnormal elastic fibers. The genes of elastin and fibrillin are known to be upregulated in chronically photodamaged skin (Bernstein *et al*, 1994). *In vitro*, vitamin C downregulates the biosynthesis of elastin by fibroblasts (Davidson *et al*, 1997) at the concentration that increases the level of mRNA for collagen in the same cells. In this study, the mRNA for elastin and fibrillins was not modified by vitamin C. Glycosaminoglycans and proteoglycans also accumulate in photoaged skin (Bernstein *et al*, 1996). Decorin is a small proteoglycan closely associated with the collagen fibrils. The increased level of its mRNA in the vitamin C-treated skin is at the limit of significance. The level of accumulated solar damage estimated in the placebo-treated side of the testers does not correlate with the extent of responsiveness to the topically applied vitamin. This suggests that a reduced biosynthetic activity related to chronologic aging and/or a low tissue concentration of the vitamin are the likely targets of the topical treatment.

The excellent technical assistance of M.J. Nix and T. Heyeres in the preparation of RNA samples and reverse transcription–PCR assays is gratefully acknowledged. The help of A. Albert for the statistical analysis of the data has been greatly appreciated. We thank C. Clabeck for the preparation of the manuscript. This work was supported in part by grants of the Belgian FRSM and the Faculty of Medicine of the University of Liège.

REFERENCES

Banhegyi G, Braun L, Csala M, Puskas F, Mandl J: Ascorbate metabolism and its regulation in animals. *Free Rad Biol Med* 23:793–803., 1997

Bartley W, Krebs HA, O'Brien JRP: Vitamin C requirement of human adults. *Med Res Counc Spec Rep Ser* 280:1–179, 1953

Bergman I, Loxley R: Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal Chem* 35:1961–1965, 1963

Bernstein EF, Chen YQ, Tamai K, et al: Enhanced elastin and fibrillin gene expression in chronically photodamaged skin. *J Invest Dermatol* 103:182–186, 1994

Bernstein EF, Underhill CB, Hahn PJ, Brown DB, Uitto J: Chronic sun exposure alters both the content and distribution of dermal glycosaminoglycans. *Br J Dermatol* 135:255–262, 1996

Colige A, Li S-W, Sieron AL, Nusgens BV, Prockop DJ, Lapière CM: cDNA cloning and expression of bovine procollagen I N-proteinase: a new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. *Proc Natl Acad Sci USA* 94:2374–2379, 1997

Colven RM, Pinnell SR: Topical vitamin C in aging. *Clin Dermatol* 14:227–234, 1996

Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV: Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol* 136:729–743, 1997

Darr D, Combs S, Dunston S, Manning T, Pinnell S: Topical Vitamin-C protects porcine skin from ultraviolet radiation-induced damage. *Br J Dermatol* 127:247–253, 1992

Darr D, Dunston S, Faust H, Pinnell S: Effectiveness of antioxidants (Vitamin C and E) with and without sunscreens as topical photoprotectants. *Acta Derm Venereol (Stockh)* 76:264–268, 1996

Davidson JM, LuValle PA, Zoia O, Quaglino D, Giro MG: Ascorbate differentially regulates elastin and collagen biosynthesis in vascular smooth muscle cells and skin fibroblasts by pretranslational mechanisms. *J Biol Chem* 272:345–352, 1997

Epstein EH: [alpha1 (III)]$_3$ Human Skin Collagen. Release by pepsin digestion and preponderance in fetal life. *J Biol Chem* 249:3225–3231, 1974

Fisher GJ, Talwar HS, Lin JY, Voorhees JJ: Molecular mechanisms of photoaging in human skin in vivo and their prevention by all-trans retinoic acid. *Photochem Photobiol* 69:154–187, 1999

Freeman WM, Walker SJ, Vrana KE: Quantitative RT-PCR: Pitfalls and potential. *Biotechniques* 26:112–125, 1999

Geesin JC, Darr D, Kaufman R, Murad S, Pinnell SR: Ascorbic acid specifically increases type I and type III procollagen messenger RNA levels in human skin fibroblast. *J Invest Dermatol* 90:420–424, 1988

Gross J: On the significance of the soluble collagens. In: Page IH (ed.). *Connective Tissue, Thrombosis and Atherosclerosis*. New York: Academic Press, 1959, pp 77–95

Hamalainen ER, Jones TA, Sheer D, Taskinen K, Pihlajaniemi T, Kivirikko KI:

Molecular cloning of human lysyl oxidase and assignment of the gene to chromosome 5q23.3–31.2. *Genomics* 11:508–516, 1991

Hirschmann JV, Raugi GJ: Adult scurvy. *J Am Acad Dermatol* 41:895–906, 1999

Kivirikko KI, Prockop DJ: Enzymatic hydroxylation of proline and lysine in protocollagen. *Proc Nat Acad Sci USA* 57:782–789, 1967

Lapière ChM, Piérard GE: Skin procollagen peptidase in normal and pathologic conditions. *J Invest Dermatol* 62:582–586, 1974

Legrand Y, Lapière ChM, Pignaud G, Caen J: Microméthode d'extraction du collagène à partir de biopsie de peau humaine. *Pathol Biol* 17:991–993, 1969

Levene CI: Ascorbic acid and collagen synthesis in cultured fibroblasts. *Ann N Y Acad Sci* 258:288–306, 1975

Levine M, Rumsey SC, Daruwala R, Park JB, Wang Y: Criteria and recommendations for vitamin C intake. *JAMA* 281:1415–1423, 1999

Li SW, Sieron AL, Fertala A, Hojima Y, Arnold WV, Prockop DJ: The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1. *Proc Natl Acad Sci USA* 93:5127–5130, 1996

Lyons BL, Schwarz RI: Ascorbate stimulation of PAT cells causes an increase in transcription rates and a decrease in degradation rates of procollagen mRNA. *Nucleic Acids Res* 12:2569–2579, 1984

Millis AJ, Hoyle TM, McCue HM, Martini H: Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts. *Exp Cell Res* 201:373–379, 1992

Nagase H, Woessner JF: Matrix metalloproteinases. *J Biol Chem* 274:21491–21494, 1999

Nimni ME, Bavetta LA: Collagen synthesis and turnover in the growing rat under the influence of methylprednisolone. *Proc Soc Exp Biol Med* 117:618–624, 1964

Padh H: Cellular functions of ascorbic acid. *Biochem Cell Biol* 68:1166–1173, 1990

Pauling L: Effect of ascorbic acid on incidence of spontaneous mammary tumors and UV-light-induced skin tumors in mice. *Am J Clin Nutr* 54:1252S–1255S, 1991

Peterkofsky B: the effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Arch Biochem Biophys* 152:318–328, 1972

Phillips CL, Tajima S, Pinnell SR: Ascorbic acid and transforming growth factor-beta1 increase collagen biosynthesis via different mechanisms—coordinate regulation of proalpha1(I) and proalpha1(III) collagens. *Arch Biochem Biophys* 295:397–403, 1992

Pinnell SR, Murad S, Darr D: Induction of collagen synthesis by ascorbic acid. *Arch Dermatol* 123:1684–1686, 1987

Ponec M, Weerheim A, Kempenaar J, Mulder AA, Gooris GS, Bouwstra J, Mommaas AM: The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. *J Invest Dermatol* 109:348–355, 1997

Ramirez F, Pereira L: The fibrillins. *Int J Biochem Cell Biol* 31:255–259, 1999

Rosenbloom J, Harsch M, Jimenez S: Hydroxyproline content determines the denaturation temperature of chick tendon collagen. *Arch Biochem Biophys* 158:478–484, 1973

Sauberlich HE: Pharmacology of vitamin C. *Annu Rev Nutr* 14:371–391, 1994

Schectman G: Estimating ascorbic acid requirement for cigarette smokers. *Ann N Y Acad Sci* 686:335–345, 1993

Schwarz RI: Procollagen secretion meets the minimum requirements for the rate-controlling step in the ascorbate induction of procollagen synthesis. *J Biol Chem* 260:3045–3049, 1985

Seite S, Colige A, Piquemal-Vivenot P, et al: A full-UV spectrum absorbing daily use cream protects human skin against biological changes occurring in photoaging. *Photodermatol Photoimmunol Photomed* 16:147–155, 2000

Sohal RS, Weindruch R: Oxidative stress, caloric restriction and aging. *Science* 273:59–63, 1996

Tebbe B, Wu SL, Geilen CC, Eberle J, Kodelja V, Orfanos CE: L-ascorbic acid inhibits UVA-induced lipid peroxidation and secretion of IL-1 alpha and IL-6 in cultured human keratinocytes in vitro. *J Invest Dermatol* 108:302–306, 1997

Thomas DP: Sailors scurvy and science. *J R Soc Med* 90:50–54, 1997

Utoguchi N, Ikeda K, Saeki K, et al: Ascorbic acid stimulates barrier function of cultured endothelial cell monolayer. *J Cell Physiol* 163:393–399, 1995

Varani J, Warner RL, Gharaee-Kermani M, et al: Vitamin A: antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J Invest Dermatol* 114:480–486, 2000

Welch RV, Wang Y, Crossman A Jr, Park JB, Kirk KL, Levine M: Accumulation of vitamin C (ascorbate) and its oxidized metabolite dehydroascorbic acid occurs by separate mechanisms. *J Biol Chem* 270:12584–12592, 1995

Wlaschek M, Briviba K, Stricklin GP, Sies H, Scharffetter-Kochanek K: Singlet oxygen may mediate the ultraviolet A-induced synthesis of interstitial collagenase. *J Invest Dermatol* 104:194–198, 1995