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Sodium L-ascorbate enhances elastic fibers deposition by fibroblasts from normal and pathologic human skin

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ABSTRACT

Background: Vitamin C (L-ascorbic acid), a known enhancer of collagen deposition, has also been identified as an inhibitor of elastogenesis. Objective Present studies explored whether and how the L-ascorbic acid derivative (+) sodium L-ascorbate (SA) would affect production of collagen and elastic fibers in cultures of fibroblasts derived from normal human skin and dermal fat, as well as in explants of normal human skin, stretch-marked skin and keloids.Methods Effects of SA on the extracellular matrix production were assessed quantitatively by PCR analyses, western blots, biochemical assay of insoluble elastin and by immuno-histochemistry. We also evaluated effects of SA on production of the reactive oxygen species (ROS) and phosphorylation of IGF-I and insulin receptors. Results SA, applied in 50–200 μM concentrations, stimulates production of both collagen and elastic fibers in all tested cultures. Moreover, combination of SA with a proline hydroxylase inhibitor induces a beneficial remodelling in explants of dermal scars, resulting in the inhibition of collagen deposition and induction of new elastogenesis. Importantly, we revealed that SA stimulates elastogenesis only after intracellular influx of non-oxidized ascorbate anions (facilitated by the sodium-dependent ascorbate transporter), that causes reduction of intracellular ROS, activation of c-Src tyrosine kinase and the enhancement of IGF-1-induced phosphorylation of the IGF-1 receptor that ultimately triggers elastogenic signalling pathway. Conclusion Our results endorse the use of this potent stimulator of collagen and elastin production in the treatment of wrinkled and stretch-marked skin. They also encourage inclusion of SA into therapeutic combinations with collagenogenesis inhibitors to prevent formation of dermal scars and keloids.

1. Introduction

As the major components of dermal extracellular matrix, collagen- and elastic fibers provide skin with mechanical strength and resiliency, respectively [1]. Elastic fibers are composed of a microfibrillar scaffold containing several glycoproteins and a core made of cross-linked elastin [2]. They are mainly produced during the second half of foetal development and in early childhood [3]. They do not undergo any extensive turnover and are supposed to last one's lifetime [4]. However, aging processes determined by a combination of genetics and environmental factors, as well as local inflammation and mechanical injuries, cause activation of multiple proteases and consequent loss of skin elasticity [1,5,6]. The extensive loss of elastic fibers clearly contributes to the formation of wrinkles and stretch marks because they cannot be spontaneously repaired or adequately replaced [7,8]. Although new ECM produced during the healing of dermal wounds contains a small amount of elastic fibers, hypertrophic scars and keloids practically do not contain elastic fibers [9–11]. The initiation of elastin gene transcription can be positively regulated by such endogenous factors as glucocorticoids [12], IGF-1 [13], insulin [14], TGF-β [15], and aldosterone [16], along with a few exogenous factors such as

Abbreviations: AA, L-ascorbic acid; CM-H2DCFDA, 5-chloromethyl-2,7-dichlorodihydro-fluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMOG, dimethyloxalylglycine; FBS, fetal bovine serum; IGF-1, insulin-like growth factor-1; IGF-IR, insulin-like growth factor-1 receptor; PPP, picropodophylin; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(3,4-d)pyrimidine, ROS, reactive oxygen species; SA, (+)sodium L-ascorbate; SVCTs, sodium-dependent vitamin C transporters.

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dexamethasone [17], retinoids [18] and ferric ions [19]. In contrast, tumor necrosis factor-α [20], interleukin-1β [21], basic fibroblast growth factor [22] and vitamin D3 [23] have been shown to down-regulate elastin gene expression. Interestingly, L-ascorbic acid (AA), a potent stimulator of collagen production [24–26], has also been listed as an inhibitor of elastin deposition. It has been suggested that AA may destabilize tropoelastin mRNA [27–29] and cause overwhelmed hydroxylation on prolyl/lysyl residues of tropoelastin molecules, thereby promoting their intracellular accumulation and inhibiting their secretion [30].

Our present studies explored the elastogenic potential of the L-ascorbic acid derivative (+) sodium L-ascorbate (SA) in experimental models utilizing primary cultures of skin fibroblasts, fat-derived fibroblasts and cultures of dermal explants derived from normal and pathologic human skin. The obtained results revealed a peculiar chain of cellular mechanisms, in which very low concentrations of SA stimulate elastogenesis.

2. Materials and methods

2.1. Materials

In all described experiments, we used (+)-sodium L-ascorbate (CAS 134-03-2) from Sigma–Aldrich (St. Louis, MO) prepared in the form of 99.0% pure powder suitable for cell culture (A4034). However, in several pilot experiments we also tested a preparation of SA from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (sc-215877) and found that both SA preparations obtained from different sources produced comparable results. All other chemical-grade reagents, L-ascorbic acid, human insulin, human IGF-I, inhibitor of IGF-1 receptor-I PPP, and inhibitor of C-Src kinse PP2 were from Sigma–Aldrich (St. Louis, MO). Probenecid was from ICN Biomedicals Inc. (Aurora, OH). The prolyl hydroxylase inhibitor, DMOG, was from Cayman Chemical (Ann Arbor, MI). The aldosterone synthetase inhibitor, 4-fluoro-*N*-(3-pyridin-3-yl) benzamide, was from Chem Div, Inc. (San Diego, CA). The DMEM, FBS and other cell culture products were acquired from GIBCO Life Technologies (Burlington, ON).

2.2. Biopsies and experimental design

The approval from the Medical Ethical Review Board and patient informed consents were obtained for all described studies that used small fragments of skin excess collected during plastic surgery procedures. Guidelines for the protection of human subjects of the Department of Health and Human Services and of the Declaration of Helsinki Principles were followed in obtaining tissues for this investigation. In order to eliminate a possible effects of regional skin heterogeneity, in all described experiments, we utilized skin biopsies derived from the identical quadrant of lower abdominal region of 6 normal subjects, 6 patients with stretch-marked skin, and 5 patients with abdominal keloids. All donors were 25–37-year-old Caucasian females. In all biochemical and morphological assays, quadruplicate samples derived from each experimental group were tested, in three separate experiments. Mean and standard deviations (SD) were calculated for each experimental group, and statistical analyses were carried out by ANOVA, followed by Bonferroni's test comparing selected groups, or by *t*-test, as appropriate. *P*-value of less than 0.05 was considered significant.

2.3. Cell cultures

Fibroblasts initially grew out from the explants of these full thickness skin biopsies and were maintained as previously described [16,17]. The primary cultures of fat-derived fibroblasts obtained from Thermogenesis (Rancho Cordova, CA) were also tested. In all described experiments, 2–4 passages of both kinds of fibroblasts were used. In experiments aimed at assessing ECM production, cells were initially plated in 35 mm culture dishes (100,000 cells/dish). Confluent cultures were then maintained for indicated times (18–72 h) in (DMEM) medium, in the presence and absence of 5% FBS. Different tested reagents were added 1 h before treatments with SA.

2.4. Immuno-staining

The 72 h-old cultures maintained in the presence and absence of indicated reagents were either fixed in cold 100% methanol at −20 °C (for detection of elastin) or in 4% paraformaldehyde at room temperature (for detection of collagen I). The multiple parallel cultures were then incubated with 10 μg/ml of polyclonal antibody to tropoelastin (Elastin Products, Owensville, MI), or polyclonal antibody to collagen type I (Chemicon, Temecula, CA). Cultures were then incubated with the respective fluorescein-conjugated goat anti-rabbit, goat anti-mouse, or rabbit anti-goat secondary antibodies. Nuclei were counterstained with propidium iodide (Sigma, Sigma, St. Louis, MO). All cultures were then examined with a Nikon Eclipse E1000 microscope attached to a cooled CCD camera (QImaging, Retiga EX) and analyzed with the computer-generated morphometric analysis system, in which the Image-Pro Plus software (Media Cybernetics, Silver Springs, MD) estimates the proportion of areas marked with green fluorescence, in relation to the entire (1 square mm) analyzed field, as previously described [14,16,19].

2.5. One step PCR and quantitative real time PCR analyses

Confluent cultures of skin fibroblasts were treated for 18 h with or without the reagents of interest for different periods of time as indicated in the figure legend. At the end of the treatments, total RNA was extracted from individual cultures using the RNeasy Mini Kit, and the one step PCR or quantitative real time PCR reactions were set up with the RT-PCR Kit, according to the manufacturer's (Qiagen, Mississauga, ON) instructions, using the previously described primers and conditions [17]. The amounts of tropoelastin mRNA obtained from triplicate cultures were normalized by levels of 18 s mRNA and then analyzed by the Comparative Ct Method, using software from Applied Biosystems and normalized to the amounts of GAPDH mRNA or 18 s mRNA.

2.6. Western blots

At the end of indicated experiments, 24 h-old separate cultures were lysed with NP-40 buffer containing a cocktail of broad-spectrum inhibitors of proteinases and phosphatases. The 50 μg aliquots of protein extract were then resolved by SDS-PAGE gel (4–12% gradient) in reducing conditions and analyzed by Western blot with antibodies indicated in figure legends, as previously described [19]. Initial blots were also re-probed with monoclonal anti-β-actin antibody (Cell Signaling Technology Inc., Danvers, MA) to confirm the equal protein loading. The degree of expression was measured by densitometry.

2.7. Quantitative assays of insoluble elastin

Cultures of fibroblasts (plated in 35 mm culture dishes at 100,000 cells/dish) were maintained for 72 h with 2 μCi of [^{3}H]-valine/ml (Amersham Biosciences Ltd. Oakville, Canada), in the presence and absence of the indicated treatments. At the end of each experiment, the levels of metabolically labeled NaOH-insoluble elastin present in individual cultures were assayed and normalized per their DNA content, as previously described [31,32].