



## 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.

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### 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-dichloro-dihydro-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, picropodophyllin; 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- $\alpha$  [20], interleukin-1 $\beta$  [21], basic fibroblast growth factor [22] and vitamin D3 [23] have been shown to downregulate 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 kinase 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 [<sup>3</sup>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].

## 2.8. Immuno-precipitation

To evaluate the levels of IGF-I receptor or insulin receptor phosphorylation, cultures maintained either in serum-free medium or in medium with 2% FBS were incubated for 15 min in the presence or absence of 100  $\mu$ M SA or 50 ng/ml IGF-1 with or without 30 min pretreatment with 0.5  $\mu$ M PPP or 10  $\mu$ M PP2. At the end of each experiment, cells were submerged in the lysis buffer containing a broad-range phosphatase inhibitor. The polyclonal antibodies recognizing either the  $\beta$  subunits of the IGF-IR or the  $\beta$  subunit of the insulin receptor were immobilized on separate sets of the IgG-bearing magnetic beads (Invitrogen Canada Inc., Burlington, ON) and then incubated for 1 h with the aliquots of the cell lysates containing 400  $\mu$ g of protein as described in the manufacturer protocol. The beads bearing the resulting immuno-precipitation products were re-suspended in a sample buffer and the released proteins were resolved with SDS-PAGE and subjected to Western blotting with a monoclonal anti-p-Tyr (PY99) antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and then with HRP-conjugated goat anti-mouse secondary antibody.

## 2.9. Quantification of intracellular free radicals-reactive oxygen species (ROS)

To assess the levels of ROS, quadruplicate cultures of normal skin fibroblasts were exposed to 10  $\mu$ M of ROS-sensitive CM-H<sub>2</sub>DCFDA fluorescent probe (Molecular Probes, Eugene, OR) for 30 min. This probe passively diffuses into the cell interior, and only upon oxidation is a fluorescent product released that can be visualized under a fluorescent microscope or captured by flow cytometry when excited at 480 nm [19,33,34]. Fibroblasts were then maintained for 30 min in the presence or absence of 400  $\mu$ M probenecid, then incubated either for 2 or 24 h with and without 100  $\mu$ M AA or 100  $\mu$ M SA. At the end of these periods the images were captured using a fluorescent microscope under identical parameters of contrast and brightness. The ROS production was also assessed by flow cytometry ( $\lambda$  excitation 480 nm;  $\lambda$  emission 520 nm), using FACSCalibur, Beckton Dickinson Instrument.

## 2.10. Organ cultures of skin explants

Fragments of normal skin, stretch-marked skin, and dermal scars collected during plastic surgery procedures were cut into multiple 4 mm<sup>2</sup> pieces and maintained for 7 days in DMEM medium containing 5% FBS, in the presence or absence of 200  $\mu$ M SA alone or in combination with 200  $\mu$ M DMOG as described in figure legends. The parallel quadruplicate explants from each experimental group were additionally maintained in the presence 2  $\mu$ Ci of [<sup>3</sup>H]-valine/ml and then subjected to the assay of insoluble elastin. The parallel explants from each experimental groups were also evaluated after pentachrome Movat's staining [35] which allows for clear (black) marking of elastic fibers. In each experimental group, 50 transversal sections derived from quadruplicate explants were analyzed with the computer-generated morphometric analysis system, in which the Image-Pro Plus software (Media Cybernetics, Silver Springs, MD) estimated the proportion of areas occupied by the black elastic fibers, in relation to the entire (1 square mm) analyzed field, as previously described [14,16,19,35].

## 2.11. Statistical evaluation

In all above described assays, the mean and SD were calculated. Student's *t*-test was used to calculate the *P*-value. The *P*-value of 0.05 was considered significant.

## 3. Results

### 3.1. SA induces the deposition of elastic fibers in cultures of human skin-derived fibroblasts

We found that only very small concentrations of SA, ranging from 50  $\mu$ M to 200  $\mu$ M, significantly stimulated production of immuno-detectable elastic fibers in 72 h-old cultures of normal dermal fibroblasts. In contrast, higher concentrations of SA did not further stimulate deposition of elastic fibers (400  $\mu$ M), and even induced a clear inhibition of elastogenesis (800  $\mu$ M SA). We also established that treatments of parallel cultures, either with 100–200  $\mu$ M NaCl or with a mixture of 100  $\mu$ M of NaCl and 100  $\mu$ M of AA, did not cause any up-regulation in elastic fibers deposition (Fig. 1A, upper panels). Moreover, treatment of parallel cultures with 100  $\mu$ M AA alone that stimulated collagen deposition, completely inhibited their elastogenesis. While causing remarkable up-regulation in the net deposition of new elastic fibers, treatment with 100  $\mu$ M SA also induced a more potent up-regulation in the deposition of the immuno-detected collagen fibers than 100  $\mu$ M AA (Fig. 1B, upper panels).

We have also established that addition of the prolyl-hydroxylase inhibitor, DMOG [36] along with 100  $\mu$ M SA to cultured fibroblasts inhibited the deposition of collagen fibers, but did not diminish the enhanced production of elastic fibers observed in 3 day old cultures. However, all cultures that were maintained for 7 days and received 6 daily doses of 100  $\mu$ M SA, contained more elastin than their 3 day-old counterparts. Moreover, cultures jointly treated with SA and DMOG displayed a significantly higher levels of immuno-detectable elastic fibers and levels of metabolically labelled insoluble elastin than cultures treated with SA alone (Fig. 1C).

All above results, based on morphometric evaluations of immuno-detected elastic fibers, correlated well with results from a quantitative assay of metabolically labelled insoluble elastin performed on parallel cultures (Fig. 1A–C, lower panels).

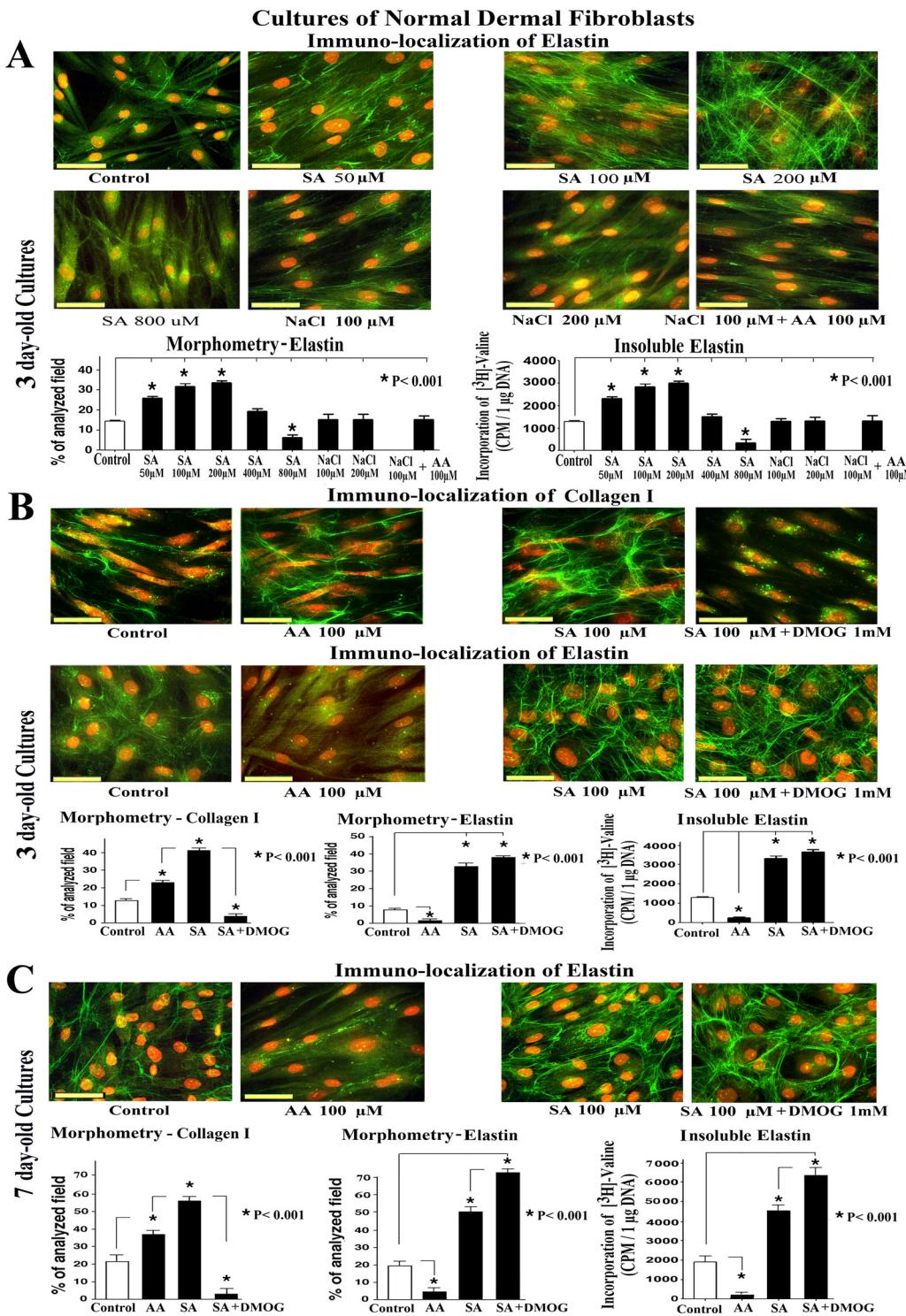
### 3.2. SA enhances deposition of collagen and elastin by cultured fat-derived fibroblasts

Morphometric evaluation of immuno-staining along with the quantitative assay of metabolically labelled insoluble elastin in parallel cultures of human fat tissue-derived fibroblasts indicated that similar concentrations of SA (50–200  $\mu$ M) induced even more potent (+22%  $\pm$ 4%) elastogenic effects (*p* < 0.01) than in cultures of dermal fibroblasts (data not shown).

### 3.3. Inhibition of the sodium-dependent vitamin C transporters eliminates SA-induced elastogenesis

Our exploration of the cellular mechanism in which SA stimulates production of elastic fibers demonstrated that treatment with 100  $\mu$ M of SA caused a significant up-regulation in levels of tropoelastin-encoding mRNA (detected either by one step PCR or by RT PCR), 18 h after addition of this compound to cultures maintained in medium with 5% FBS. This preceded an increase in the levels of newly synthesized tropoelastin (detected by western blots) in 24 h-old cultures and in levels of insoluble elastin observed in 72 h-old cultures. The results of three separate experiments in which we tested the elastogenic potential of 100  $\mu$ M SA are shown in Fig. 2A–D.

Since the salt configuration of SA molecules ensures their temporal stability in the culture medium (pH 7.4), we assumed that intact SA molecules would preferably interact with the cell surface-residing sodium-dependent vitamin C transporters (SVCTs) that can quickly transport the non-oxidized ascorbate



**Fig. 1.** Small concentrations (50–200 μM) of SA up-regulate production of both elastic and collagen fibers in cultures of normal dermal fibroblasts. In contrast, treatment with 800 μM SA inhibits elastogenesis. Treatment with 100–200 μM NaCl or with a combination of 100 μM NaCl and 100 μM AA does not induce elastogenesis. (A) Representative micrographs (upper panels) depicting immuno-detected elastic- and collagen fibers in 3 day-old cultures (upper panels), followed by the results of their morphometric evaluation and quantification of metabolically-labelled insoluble elastin (lower panels). Cell nuclei stained red with propidium iodide. (Scale bars = 15 μm). (B) Similar techniques demonstrate that 100 μM SA induces a more potent up-regulation in collagen fibers deposition than 100 μM AA, which also completely inhibits elastic fibers formation. Addition of 1 mM DMOG (proline hydroxylase inhibitor) to SA-treated cultures significantly inhibits the deposition of collagen fibers, but does not diminish the elastogenic effect of SA. (C) 7 day-old cultures contain proportionally more elastin than their 3 day-old counterparts and a net increase in the rate of new elastogenesis in parallel cultures jointly treated with SA and DMOG is significantly higher than this observed in cultures treated with SA only. Results are based on data obtained from three individual experiments, in which quadruplicate cultures were statistically evaluated and finally expressed as mean ± SD.

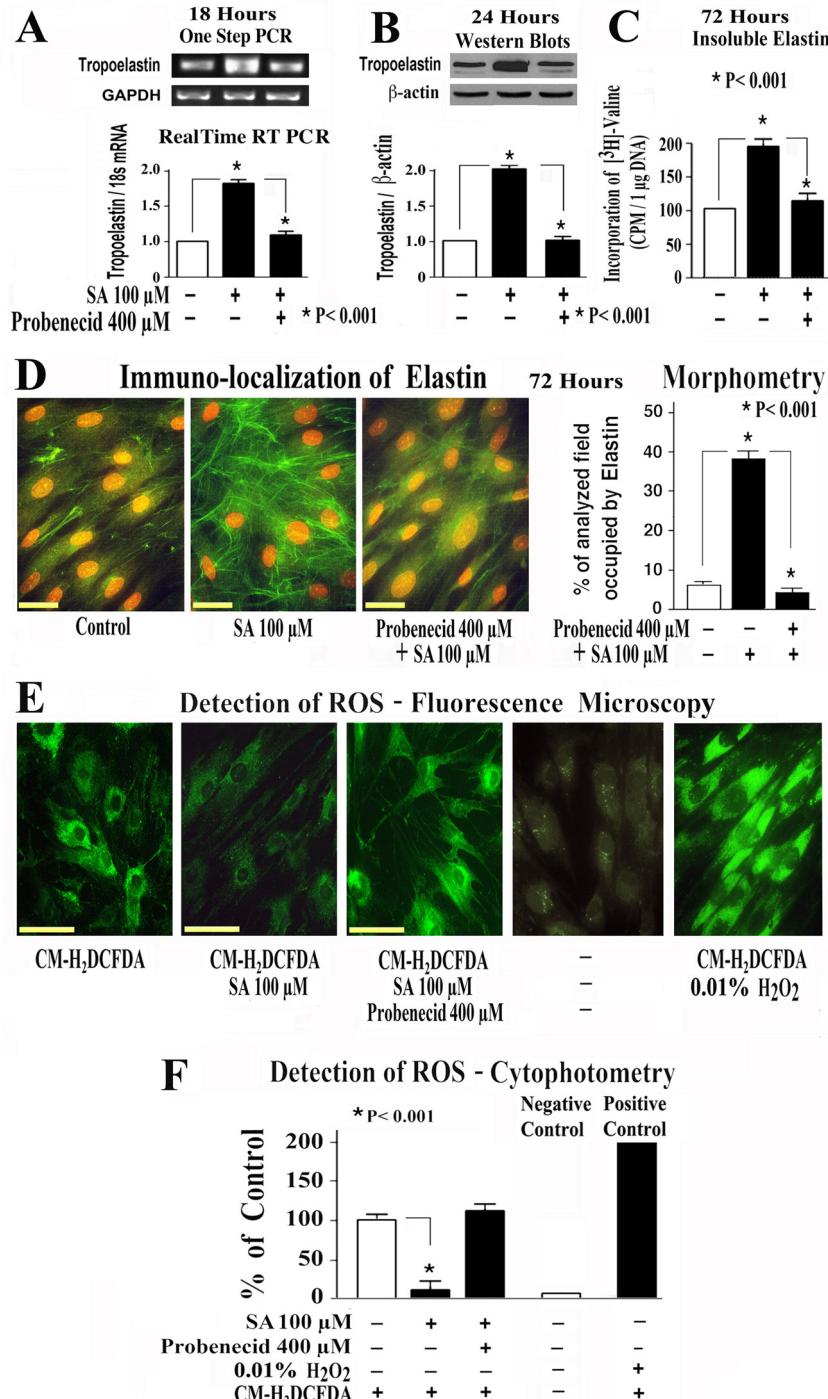
anions into the cell interior [37–39]. Therefore, we also exposed cultured skin fibroblasts to 400 μM probenecid and found that their 10 min-long pre-incubation with this SVCTs inhibitor [40] eliminated the elastogenetic effects of 100 μM SA observed on the

message, precursor protein and final product levels (Fig. 2A–D). Meaningfully, cultures maintained for 24 h with 100–400 μM probenecid alone did not demonstrate any decrease in their basic deposition of elastic fibers below the level observed in untreated

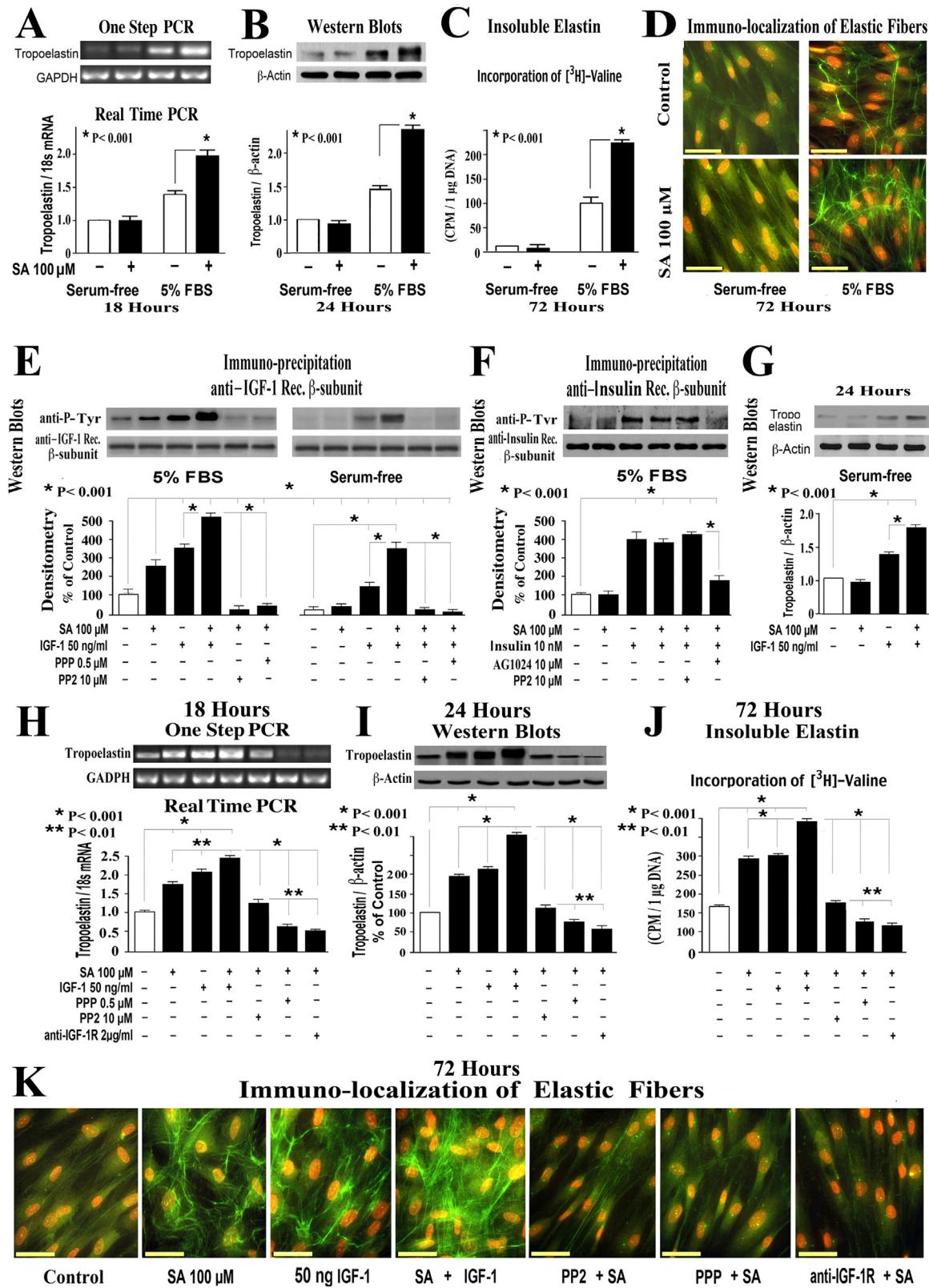
control cultures (data not shown). Together, results obtained from three independent experiments strongly suggested that quick active transportation of SA-derived, non-oxidized ascorbate anions into the cell interior may contribute to the enhancement of elastogenesis.

### 3.4. Influx of SA associates with a decrease in levels of intracellular reactive oxygen species (ROS)

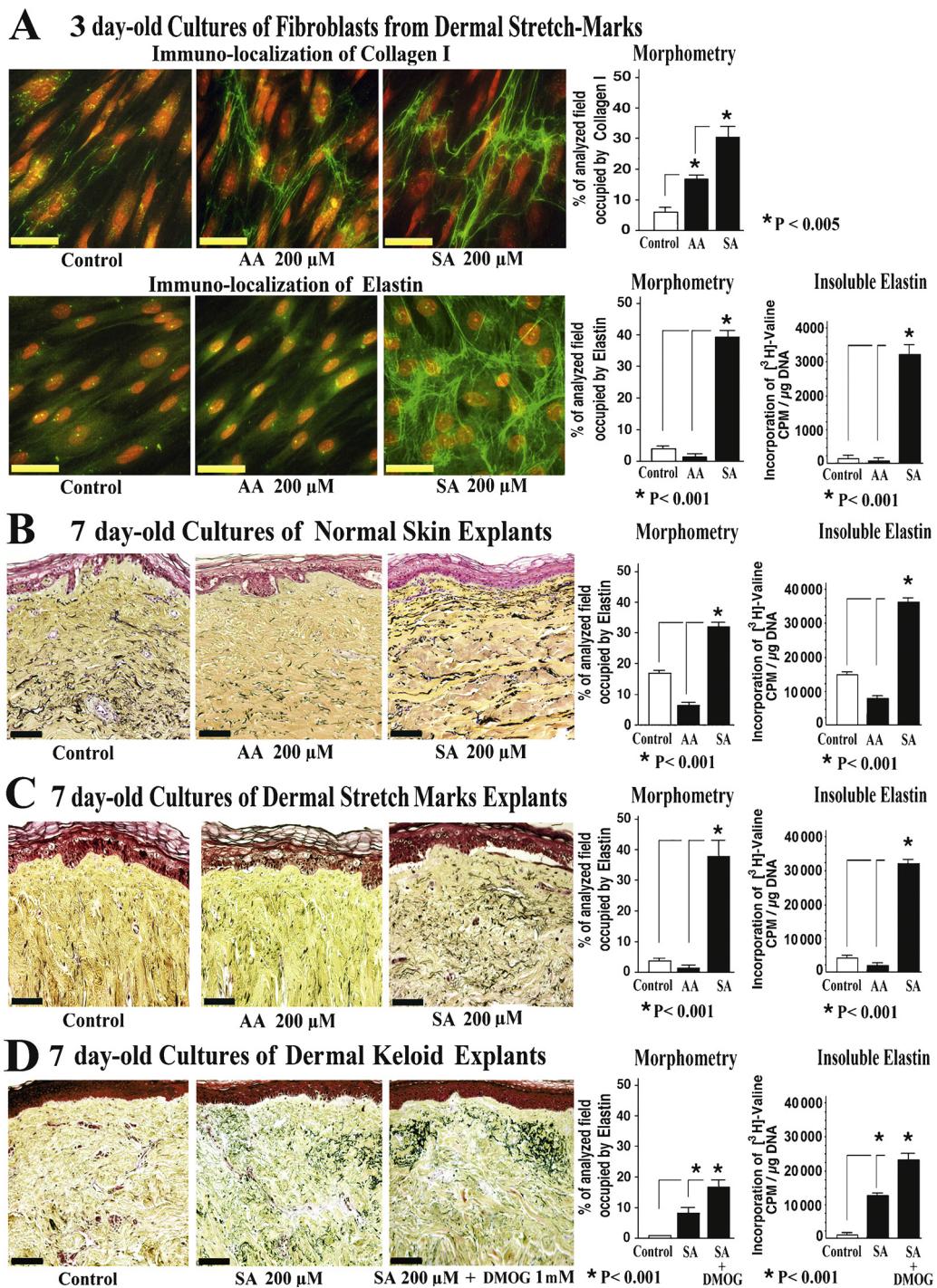
Next, we exposed fibroblasts to the ROS-sensitive fluorescent probe, CM-H<sub>2</sub>DCFDA and found that cells treated for only 2 h with



**Fig. 2.** Intracellular influx of SA-derived non-oxidized ascorbate anions causes a decrease in reactive oxygen species and contributes to the initiation of elastogenesis in cultures of dermal fibroblasts. (A) Representative images of one step RT-PCR visualizing levels of tropoelastin and GAPDH mRNAs correlating with results of quantitative real-time RT-PCR. (B) results of quantitative Western blot-based assessments of intracellular tropoelastin, followed by a quantitative assay of metabolically-labelled insoluble elastin (C) and morphometric evaluations of the immuno-detected elastic fibers (D). These results demonstrate that probenecid-dependent inhibition of the intracellular transport of SA-derived non-oxidized ascorbate anions averts induction of the elastogenic effects observed in cultures treated with SA alone. (E and F) Fibroblasts exposed to the ROS-sensitive fluorescent probe and treated for 2 h with 100  $\mu$ M SA contain significantly lower levels of ROS detected by both fluorescence microscope and flow cytometry. This effect of SA could not be observed in cultures in which the intracellular influx of SA has been inhibited by pre-incubation with probenecid. (scale bars = 15  $\mu$ m). Results (mean  $\pm$  SD) are based on data obtained from three individual experiments, in which quadruplicate cultures were exposed to indicated treatments. Exclusion of the fluorescent probe and addition of 0.01% hydrogen peroxide represent the negative and positive controls, respectively.



**Fig. 3.** SA enhances the synthetically effective elastogenic signals triggered by the IGF-1 receptor. (A) Representative images of one step RT-PCR visualizing tropoelastin and GAPDH mRNAs along with the results of quantitative real-time RT-PCR, followed by the results of quantitative assessments of western blots-detected intracellular tropoelastin (B) metabolically-labelled insoluble elastin (C) and representative images of immuno-staining (D). These results indicate that 100  $\mu$ M SA alone up-regulates elastogenesis only in cultures of dermal fibroblasts maintained in media containing 5% FBS. (E) Treatment with 100  $\mu$ M SA alone enhances the level of phosphorylation of the IGF-1R (immuno-precipitated with antibody recognizing  $\beta$  subunit of IGF-1R and detected by Western blotting with anti-phospho-tyrosine antibody) only in cultures maintained in media containing 5% FBS. However, SA enhances levels of IGF-1-induced IGF-IR phosphorylation in both tested media. These SA-induced enhancements of IGF-1R phosphorylation are eliminated in cultures pre-treated with IGF-1 receptor kinase inhibitor (PPP) or with c-Src kinase inhibitor (PP2). (F) In contrast, treatment with 100  $\mu$ M SA does not enhance the basic or insulin-induced phosphorylation of the insulin receptor. (G) Meaningfully, cultures of dermal fibroblasts, maintained in serum free media produce more tropoelastin when co-treated with IGF-1 and SA than their counterparts treated with IGF-1



**Fig. 4.** Treatment with SA up-regulates elastogenesis in cultures of fibroblasts derived from dermal stretch marks, and in cultured explants derived from biopsies of stretch-marked skin and dermal keloids. Explants of cultured keloids, jointly treated with SA and DMOG, contain more elastic elastin than parallel explants treated with SA alone. (A) Representative micrographs of immuno-detected collagen- and elastic fibers (left site panels) and results of their morphometric evaluations, followed by quantification of insoluble elastin (right site panels) in 3 day-old cultures. (B-D) Micrographs of Movat's pentachrome-stained sections of dermal explants derived from normal skin, stretch marks and keloids, cultured for 7 days (left site panels), accompanied with results of morphometric evaluations of elastic fibers and quantitative evaluation of insoluble elastin (right site panels), (scale bars = 15  $\mu\text{m}$ ). Results (mean  $\pm$  SD) are based on data obtained from three experiments utilizing biopsies from 5 individuals, in which quadruplicate cultures were exposed to indicated treatments.

100  $\mu\text{M}$  SA contained significantly lower levels of ROS than could be detected by either fluorescence microscopy or flow cytometry (Fig. 2E,F). This effect was not observed in cultures in

which the intracellular influx of SA-derived, non-oxidized ascorbate had been inhibited by pre-incubation with probenecid. Parallel cultures treated for 2 h with 100  $\mu\text{M}$  AA, did not display

alone. (H-K) Pre-incubation with either PP2, PPP or anti-IGF-1R-blocking antibody abolishes all elastogenic effects that could be observed in indicated times in cultures of dermal fibroblasts treated with IGF-1 and/or SA. Results (mean  $\pm$  SD) are based on data obtained from three individual experiments, in which quadruplicate cultures were exposed to indicated treatments. (scale bars = 15  $\mu\text{m}$ ).

any decrease in ROS contents, as compared with untreated counterparts (data not shown).

### 3.5. SA induces enhancement of the primary elastogenic signals triggered by IGF-1 receptor

Surprisingly, we also found that the elastogenic effects of SA, evident in 18–72 h-old cultures maintained in the presence of FBS, could not be observed in parallel cultures maintained in serum-free medium (Fig. 3A–D). This result suggested that SA might only enhance elastogenic signals triggered by some other factor(s) present in the serum. Therefore, we tested whether addition of SA would enhance the basic effects of selected elastogenic stimulators; corticosteroids, TGF- $\beta$ 1 and IGF-1. Results of pilot experiments indicated that the addition of 100  $\mu$ M SA did not further enhance the increase in elastogenesis, induced by 1  $\mu$ M dexamethasone or 1 ng/ml of TGF- $\beta$ 1 (data not shown), but significantly up-regulated the levels of elastin deposition induced by 50 ng/ml of IGF-1. Therefore, we concentrated on exploring the putative mechanism by which SA would enhance the IGF-1-induced elastogenic signalling pathway.

The results of the next series of experiments (presented in Fig. 3E) revealed that addition of 100  $\mu$ M SA to fibroblasts maintained in the presence of 5% FBS (containing 2–6 ng IGF-1/ml), remarkably enhanced levels of IGF-1R phosphorylation (immuno-precipitated with antibody recognizing the  $\beta$  subunit of IGF-1R and detected on Western blot with anti-phosphotyrosine antibody). In contrast, fibroblasts maintained in serum-free medium did not exhibit any increase in IGF-1R phosphorylation in response to treatment with the same dose of SA. The possibility of the super-activation of IGF-1R by SA was also endorsed by the fact that additions of 100  $\mu$ M SA further enhanced levels of phosphorylated IGF-1R in parallel cultures maintained in both tested media, when treated with 50 ng/ml of IGF-1. These data suggested that SA-derived, non-oxidized ascorbate ions facilitated interactions between the small concentrations of IGF-1 and the IGF-1R that ultimately enhanced its phosphorylation.

Since this SA-dependent enhancement of IGF-1R phosphorylation was eliminated in cultures pretreated with either the c-Src kinase inhibitor (PP2) [41] or with inhibitor of IGF-1R tyrosine kinase (PPP) [42], we concluded that c-Src tyrosine kinase activity is required for the execution of the SA-dependent mechanism leading to the super-activation of IGF-1R. In contrast, treatment with SA did not enhance the insulin-dependent phosphorylation of the highly homologous insulin receptor (Fig. 3F). We also showed that the insulin-dependent phosphorylation of insulin receptor, that could be specifically eliminated by pretreatment with 10 nM of AG 1024, was not diminished in cultures pretreated with PP2.

Importantly, we have also established that co-treatment with and 100  $\mu$ M SA further enhanced the IGF-1-induced synthesis of tropoelastin, detected by in 24 h-old cultures maintained in serum free medium (Fig. 3G). Then, we documented that treatment of dermal fibroblasts (maintained in the presence of 5% FBS) with 100  $\mu$ M SA alone induced a similarly strong elastogenic effect as treatment with 50 ng/ml of exogenous IGF-1. Moreover, cultures jointly treated with IGF-1 (50 ng/ml) and SA (100  $\mu$ M) displayed even higher levels of tropoelastin mRNA, intracellular tropoelastin, insoluble elastin, and immuno-detected elastic fibers than their counterparts treated with either compound on its own. Meaningfully, SA did not trigger elastogenic effects in cultures pretreated with PP2, PPP or with anti-IGF-1R-blocking antibody (Fig. 3H–K). Furthermore, we have established that inhibition of other kinases contributing to the IGF-1-induced signalling pathway; phosphatidylinositol 3-kinase; (by LY294002)

or cyclin-dependent kinase-2 (by CVT313) [37] eliminated elastogenic effects of 100  $\mu$ M SA (data not shown).

### 3.6. SA ameliorates the poor deposition of collagen and elastic fibers observed in monolayer cultures of dermal fibroblasts and organ cultures of explants derived from dermal stretch marks

Our analysis of yet another experimental model of cultured fibroblasts derived from dermal stretch marks revealed that the treatment with 200  $\mu$ M SA induced a significant up-regulations in their deposition of both, collagen- and elastic fibers. Meaningfully, the treatment of parallel cultures with 200  $\mu$ M AA caused a selective inhibition of new elastogenesis (Fig. 4A).

We then explored the effects of SA in cultured explants of biopsies derived from normal human skin and from dermal stretch marks. Results of morphometric analysis of histological sections stained with pentachrome Movat's method, as well as the quantitative assay of metabolically-labelled insoluble elastin, indicated that 7 day long daily treatment with 200  $\mu$ M SA not only enhanced deposition of new elastic fibers in normal skin explants (Fig. 4B), but also restored the practically non-existent elastogenesis in the parallel cultures of explants derived from stretch marks (Fig. 4C). In contrast, parallel explants treated with 200  $\mu$ M AA demonstrated only up-regulation in the deposition of collagen (detected by Movat's method).

### 3.7. SA induces enhancement of elastic fibers deposition in cultured explants of dermal keloids

We have also established that treatment with SA induced beneficial remodelling of cultured explants of rigid dermal keloids. Both histochemistry and quantification of insoluble elastin indicated that 200  $\mu$ M SA initiated production of new elastic fibers that appeared in their mostly collagenous extracellular matrix. Moreover, we found that explants jointly treated with SA and DMOG, which blocks collagen deposition [36], demonstrated further up-regulation in the net deposition of elastic fibers (Fig. 4D). We therefore conclude that the DMOG dependent inhibition of prolyl hydroxylase effectively suppressed the fibrotic phenotype of keloid forming fibroblasts and concomitantly contributed to the enhancement of their SA-induced elastogenic capability.

## 4. Discussion

Once we established that unlike 50–200  $\mu$ M of SA, the comparable concentrations of NaCl or AA did not induce elastogenesis, we concluded that a more stable salt conformation of SA must be responsible for triggering of the described elastogenic effects. Moreover, the additional finding that the elastogenic effects of 100  $\mu$ M SA could not be observed in cultures pre-treated with the SVCTs inhibitor (probencid), further suggested that these cell membrane-residing transporters, first interact with intact SA molecules and then, after their activation by sodium cations, quickly transport the free ascorbate anions into the cell interior. It is also conceivable that such translocation of ascorbate anions likely prevents their imminent extracellular oxidization and conversion to dehydro-ascorbate [37,38]. Since we also found that a higher concentration of SA (400  $\mu$ M) did not further stimulate deposition of elastic fibers, and that treatment with 800  $\mu$ M SA induced a clear inhibitory effect, comparable to those induced by treatment with 100–400  $\mu$ M AA, we concluded that high concentrations of SA likely overwhelm the physiological capacity of the existing sodium-dependent vitamin C transporters. Therefore, those SA molecules that could not immediately interact with SVCTs also dissociate. Consequently, the freed ascorbate ions, after their

extracellular oxidation and the consecutive GLUT-dependent translocation to the cell interior, can contribute to the accumulation of dehydro-ascorbate, causing heightened hydroxylation of prolyl/lysyl residues of tropoelastin molecules [30], and deteriorating their chance for normal cross-linking and secretion.

The additional observation that elastogenic effects of 100 µM SA could be achieved only in cultures maintained in the presence of at least 5% FBS allowed for yet another conclusion that SA itself may not induce elastogenesis, but enhance elastogenic signals induced by other reagent(s) present in the serum. Indeed, further results demonstrated that treatments of cultured fibroblasts with 100 µM SA significantly enhanced levels of the phosphorylated IGF-1 receptor that could be induced by either a very low concentration of endogenous IGF-1 (5–6 ng/ml), present in the 5% FBS or by 50 ng/ml of exogenous IGF-1 added to cultures maintained in both serum-free or 5% FBS-containing media. Importantly, these SA-induced enhancements of IGF-1 receptor activation were always followed by the consequent up-regulations of the consecutive stages of elastogenesis.

IGF-I is a peptide hormone that promotes growth, differentiation and matrix production in different tissues, including skin [43,44]. Binding of IGF-I to its receptor results in IGF-I receptor auto-phosphorylation that occurs after the activation of various adaptor and signalling proteins [45,46]. While the mitogenic and collagenogenic effects of IGF-I are linked to the phosphorylation of Shc adaptor protein and stimulation of the MAPK pathway [47–49] an alternative elastogenic pathway induced by IGF-1 has also been identified [13]. Studies from our laboratory additionally established that the elastogenic pathway induced after IGF-1R phosphorylation involves PI3/Akt kinases, cyclin E/cdk-2 kinases, and the site specific phosphorylation (Tyr-821) of the retinoblastoma protein that eventually causes de-repression of the elastin gene [50]. Importantly, we have also described that the efficiency of IGF-1-induced elastogenesis could be significantly enhanced by aldosterone-induced activation of c-Src kinase that, in turn, causes phosphorylation of the IGF-1R on an additional site at Tyr-1316 [47,48].

Our results also demonstrated that the addition of SA could not enhance the level of IGF-1R phosphorylation, nor induce elastogenesis in cultures maintained in the presence of the IGF-1R tyrosine kinase inhibitor (PPP) or the c-Src inhibitor (PP2). Therefore, they strongly confirmed the prerequisite involvement of c-Src and the IGF-1R-triggered pathway in SA-induced elastogenesis. Since we have additionally demonstrated that SA still enhances elastogenesis in cultures pre-incubated with the specific inhibitor of aldosterone synthesis (data not shown), we eliminated the possibility that SA would trigger local synthesis of aldosterone and in this way contribute to the activation of c-Src and enhancement of the elastogenic signal.

Importantly, we have also established that fibroblasts maintained with SA for only 2 h demonstrated a significant decrease in their level of ROS. This observation allowed for speculation that a quick transportation of SA-derived, non-oxidized ascorbate anions contributes to the scavenging of free oxygen species and a consequent down-regulation of their intracellular concentration. This, in turn, creates permissive conditions for activation of c-Src kinase that, in turn, enhances levels of IGF-1R phosphorylation initiated by IGF-1. The proposed mechanism correlates well with previously published studies reporting that heightened levels of ROS inhibit the activity of c-Src and IGF-1 receptor kinases [51,52]. Since the oxidative modifications of tropoelastin molecules may also interfere with their final cross-linking and interactions with components of the microfibrillar scaffold [53], we additionally speculate that the SA-dependent down-regulation of ROS would also enhance the proportion of newly produced tropoelastin molecules that can be successfully assembled into elastic fibers.

The translational importance of our present studies have been especially endorsed by data indicating that SA can actually up-regulate production of both collagen and elastic fibers in monolayer cultures derived from dermal stretch marks that, as we previously established, contain rather dormant fibroblasts that do not proliferate, migrate or spontaneously produce ECM [54]. Additional observation that SA penetrated into the cultured explants of full thickness biopsies of normal skin and dermal stretch marks, to induce the deposition of both collagen and elastic fibers, particularly encourages further clinical testing whether a topically applied SA would prevent formation of wrinkles, stimulate rebuilding of the lost extracellular matrix in existing stretch marks, or accelerate wound healing. Our data also endorses the future studies on use of SA for simultaneous stimulation of collagen and elastin in bioengineered constructs containing human fibroblasts isolated from the adult human skin and fat tissue.

Our claim that sodium derivatives of ascorbic acid may have significance in regenerative medicine is consistent with the empirical observations that the addition of sodium L-ascorbate, to a hyaluronan-based hydrogel containing smooth muscle cells increased the tensile strength of bioengineered artificial vessels [55]. This is also consistent with another previous study showing that disodium isostearyl 2-O-L-ascorbyl phosphate inhibited a matrix destructive enzyme, metalloproteinase-1, in cultures of human dermal fibroblasts [56].

Especially encouraging are our observations that the simultaneous application of SA and the proline hydroxylase inhibitor DMOG allowed for the up-regulation of elastic fibers deposition that is associated with the down-regulation of net collagen content in cultured explants of dermal scars. Because it has been previously claimed that the overwhelming hydroxylation on prolyl/lysyl residues of tropoelastin molecules contributes to their intracellular retention [30], we speculate that the introduced inhibition of proline hydroxylation may actually enhance tropoelastin secretion by dermal scar fibroblasts, in which collagen secretion was simultaneously inhibited by the same compound. Therefore, we propose that topical applications or intra-lesion injection of these two compounds should be also considered for prevention and treatment of disfiguring skin lesions.

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