ARTICLE

Dexamethasone normalizes aberrant elastic fiber production and collagen 1 secretion by Loeys–Dietz syndrome fibroblasts: a possible treatment?

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Loeys–Dietz syndrome (LDS) is an autosomal dominant connective tissue disorder characterized by facial dysmorphism, cleft palate, dilation of the aortic arch, blood vessel tortuosity and a high risk of aortic dissection. It is caused by mutations in the transforming growth factor β-receptor 1 and 2 (TGFβ-R1 and TGFβ-R2) genes. Fibroblasts derived from 12 Loeys–Dietz syndrome patients, six with TGFB-R1 mutations and six with TGFB-R2 mutations, were analyzed using RT-PCR, biochemical assays, immunohistochemistry and electron microscopy for production of elastin, fibrillin 1, fibulin 1 and fibulin 4 and deposition of collagen type I. All LDS fibroblasts with TGFβ-R1 mutations demonstrated decreased expression of elastin and fibulin 1 genes and impaired deposition of elastic fibers. In contrast, fibroblasts with TGFβ-R2 mutations consistently demonstrated intracellular accumulation of collagen type I in the presence of otherwise normal elastic fiber production. Treatment of the cell cultures with dexamethasone induced remarkable upregulation in the expression of tropoelastin, fibulin 1- and fibulin 4-encoding mRNAs, leading to normalization of elastic fiber production in fibroblasts with TGFβ-R1 mutations. Treatment with dexamethasone also corrected the abnormal secretion of collagen type I from fibroblasts with TGFβ-R2 gene mutations. As the organogenesis-relevant elastic fiber production occurs exclusively in late fetal and early neonatal life, these findings may have implications for treatment in early life. Further studies are required to determine if dexamethasone treatment of fetuses prenatally diagnosed with LDS would prevent or alleviate the connective tissue and vascular defects seen in this syndrome.

INTRODUCTION

Loeys–Dietz syndrome (LDS types 1A, 1B, 2A and 2B, OMIM #609192, #610380, #610168 and #608967, respectively) is a recently described autosomal dominant connective tissue disorder caused by mutations in the transforming growth factor β-receptor 1 and 2 genes (TGFβ-R1 and TGFβ-R2).[1] The TGFβ-R1 and R2 receptors belong to the transmembrane-spanning protein serine/threonine kinase family and control several cellular processes including growth inhibition, apoptosis, proliferation and extracellular matrix production.[2,3] LDS has phenotypic overlap with Marfan syndrome (MFS, OMIM #154700), caused by mutations in the FBN1 gene (OMIM #134797) but LDS patients have a higher risk of aortic dissection.[1] Life expectancy of LDS patients is significantly shortened when compared with MFS.

The most common features of LDS type 1 are hypertelorism, cleft palate with bifid uvula, aortic root aneurysm, arterial tortuosity, arachnodactyly, pectus deformity and joint laxity, with some patients having craniosynostosis, retrognathia and blue sclerae. Patients classified as LDS type 2 do not have cleft palate, craniosynostosis or hypertelorism but demonstrate features in common with Ehlers–Danlos syndrome type IV (EDS IV, OMIM#130050, vascular type) such as increased joint laxity, easy bruising, translucent skin, distended

scars and spontaneous rupture of the spleen and bowel.[4–7] However, because of the relatively small number of fully analyzed cases bearing the same individual mutations, no consensus has been reached whether the presence of particular mutations could be exclusively linked to the given feature observed in both subtypes of LDS.

The existence of significant phenotypic overlap between LDS and MFS suggests that the causative mechanism may be similar in both conditions, despite the fact that they are caused by mutations in different genes. It has been suggested that aberrant TGFβ signaling might contribute to the connective tissue pathology in LDS as it does in MFS, where the inherited deficiency of fibrillin-1 protein contributes to intensified TGFβ-induced signals.[8–10] The molecular mechanism leading to aortic dissections in LDS has not been fully characterized, but elastic fiber fragmentation and abnormal collagen deposition was demonstrated in aortic explants from LDS patients.[11]

In our study, gene expression analysis, biochemical assays, immunohistochemistry and electron microscopy (EM) were used to compare the production of four major elastic fiber components (elastin, fibrillin 1, fibulin 1 and fibulin 4) and the production of collagen type I in fibroblasts derived from 12 LDS patients with different mutations in both TGFβ-R1 and TGFβ-R2. We also explored

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whether dexamethasone, which directly interacts with the elastin gene promoter to induce transcription of tropoelastin mRNA,[12] would improve production of elastic fibers in the cultures of LDS dermal fibroblasts.

MATERIALS AND METHODS
Cell cultures

Primary cultures of dermal fibroblast derived from six LDS patients bearing different mutations of TGFβ-R1 (S241L, R487Q, C722T, T200I, M318R and G1460A) and from six patients with different TGFβ-R2 mutations (A355P, R528H, R495X, P427L, R460L and A329T) were used in this study. The fibroblasts were collected from skin biopsies taken for diagnostic purposes or surgery at the Hospital for Sick Children in Toronto, Canada and at the Johns Hopkins Medical Center, USA (donated by Dr H Dietz). The respective institutional review board approvals and patient informed consents were obtained. The guidelines for the protection of human subjects of the Department of Health and Human Services and of the Declaration of Helsinki were strictly followed. All fibroblasts (passages 1–6) were initially plated (100 000 cells/dish and maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 1% antibiotics/antimycotics, and 2% of FBS in the presence and absence of 10 μM dexamethasone (Sigma, St Louis, MO, USA). The selected dose produced an optimal elastogenic effect in cultures of normal human skin fibroblasts. It also corresponds well to transient concentration detected in the serum of patients treated with therapeutic doses of this glucocorticoid (5–15 mg/kg). The cultures were then used for the quantitative assessment of mRNAs encoding major components of elastic fibers and collagen type 1, quantification of new insoluble elastin production, for the EM and for light microscopy immunohistochemical evaluation of elastin, fibrillin 1, fibulin 1, fibulin 4 and collagen type I deposition.

One-step reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Confluent fibroblast cultures were treated with or without 10 μM dexamethasone for 24 h. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. In each experiment, 0.5 μg of total RNA was added to each one-step RT-PCR (Qiagen One-Step RT-PCR Kit), and reactions were set up according to the manufacturer's instructions in a total volume of 25 μl. The reverse transcription step was performed for human elastin, fibrillin 1, fibulin 1, fibulin 4, collagen 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reactions at 50 °C for 30 min, followed by 15 min at 94 °C. The elastin PCR reaction was performed under the following conditions: 28 cycles at 94 °C denaturation for 45 s, 63 °C annealing for 45 s, 72 °C extension for 1 min and 1 cycle at 72 °C final extension for 10 min. The fibrillin 1 PCR reaction included: 26 cycles at 94 °C denaturation for 30 s, 52 °C annealing for 30 s, 72 °C extension for 1 min and 1 cycle at 72 °C final extension for 10 min. The fibulin-1 and fibulin-4 PCR reactions included: 28 cycles at 94 °C denaturation for 30 s, 60 °C annealing for 30 s, 72 °C extension for 1 min and 1 cycle at 72 °C final extension for 10 min. The pro-α1 (collagen I) chain PCR included 20 cycles of 94 °C denaturation for 30 s, 58 °C annealing for 30 s, 72 °C extension for 10 min and 1 cycle of 72 °C final extension for 10 min. GAPDH PCR reaction included 21 cycles of 94 °C denaturation for 20 s, 58 °C annealing for 30 s, 72 °C extension for 1 min and 1 cycle of 72 °C final extension for 10 min. The primers used are listed in Table 1. The PCR products from each reaction were run on a 1% agarose gel and stained with ethidium bromide. The dose curves were also created to validate the quantitative assay of each tested product. In each presented case, three separate reactions were performed and the amounts of mRNAs encoding particular ECM component-related product were normalized to the amount of GAPDH mRNA.

Real-time RT-PCR

For comparison, the RNA samples extracted with Rneasy Mini Kit (Qiagen) from cells of normal individuals and LDS patients were subjected to the reverse transcription proceeded with 1 μg of total RNA using SuperScript II First-Strand Synthesis System (Invitrogen). The quantitative real-time PCR reactions were then performed using SYBR Green Master Mix

Table 1

Oligonucleotide primers used for RT-PCR

GAPDH	Sense	5′-TCCACCACCCTGTTGCTGTAG-3′
	Anti-sense	5′-GACCACAGTCCATGCCATCACT-3′
Tropoelastin	Sense	5′-GGTGCGGTGGTTCCTCAGCCTGG-3′
	Anti-sense	5′-GGGCCTTGATGGGATACCCCAGTG-3′
Fibrillin-1	Sense	5′-AATGTGCTTTCAATTGATG-3′
	Anti-sense	5′-ATTACCACTTGGTGTATA-3′
Fibulin-1	Sense	5′-GGAATCCAAAGAATGCAGGA-3′
	Anti-sense	5′-ACTGGTAGCCAACCATGAGG-3′
Fibulin-4	Sense	5′-ATGCGAGCAGCGCTGCTTCAA-3′
	Anti-sense	5′-AGCCCCCATGGAAGTTGACAC-3′
Collagen-I	Sense	5′-CCCACCAATCACCTGCGTACAGA-3′
	Anti-sense	5′-TTCTTGGTCGGTGGGTGACTCTGA-3′

Oligonucleotide primers used for real-time RT-PCR

GAPDH	Sense	5′-TCAACAGCGACACCCACTCC-3′
	Anti-sense	5′-TGAGGTCCACCACCCTGTTG-3′
Tropoelastin	Sense	5′-GCTGACGCTGCTGCAGCCTA-3′
	Anti-sense	5′-CAGCAAAAGCTCCACCTACA-3′
Fibrillin-1	Sense	5′-TGACTGGCCCACACGTGCATAG-3′
	Anti-sense	5′-TGACATTGACCCCTTGTTGACAGGA-3′
Fibulin-1	Sense	5′-GGAGCAGTGCTGCCACAG-3′
	Anti-sense	5′-AGCACCTCTTCACAAATGTG-3′
Fibulin-4	Sense	5′-ATGCGAGCAGCGCTGCTTCAA-3′
	Anti-sense	5′-AGCCCCCATGGAAGTTGACAC-3′

(Applied Biosystems, Foster City, CA, USA) and carried out in 7000 Sequence Detection System at 50 °C for 2 min and then at 95 °C for 10 min, followed by 40 cycles of amplification (95 °C for 15 s and 60 °C for 1 min). The primers used are listed in Table 1. In each presented case, three separate reactions were performed and the levels of particular ECM component-encoding mRNAs were normalized to the amount of GAPDH mRNA. In both 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.

Quantitative assay of the newly produced insoluble elastin

All LDS fibroblasts were grown to confluency in 30 mm dishes and then cultures were maintained for the next 3 days in fresh media (DMEM with 2% FBS) in the presence and absence of 10 μM dexamethasone. Aliquots of 20 μCi of [3H]-valine were added to each dish along with fresh media. At the end of the incubation period, the quadruplicate cultures were used for the quantitative assessment of metabolically labeled (radioactive) insoluble elastin as previously described.[13] Cell layers containing newly deposited matrix were boiled in 0.5 ml of 0.1 N NaOH for 45 min to solubilize all matrix components except elastin. The collected insoluble radioactive elastin was then solubilized by boiling in 200 μl of 5.7 N HCl for 1 h and quantified in scintillation counter. Aliquots taken from each culture were also used for DNA determination using the DNeasy Tissue System from Qiagen. Final results reflecting amounts of metabolically labeled insoluble (crosslinked) elastin in individual cultures were normalized per their DNA content and expressed as counts per min per 1 μg of DNA.

Immunostaining

The parallel cultures maintained for 7 days in the presence and absence of 10 μM dexamethasone, which was added on day 3 and day 5, were either fixed in ice-cold 100% methanol at −20 °C (for elastin or fibrillin detection) or in 4% paraformaldehyde at room temperature (for collagen detection) for 30 min and blocked with 1% normal goat serum for 1 h. The cultures were then incubated for 1 h, either with 10 μg/ml polyclonal antibody to tropoelastin or monoclonal

antibody to fibrillin 1 (both from Elastin Products, Owensville, MO, USA), 5 μg/ml antibody to fibulin 1 (Chemicon, Billerica, MA, USA), 1 μg/ml antibody to fibulin 4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with 10 μg/ml polyclonal antibody to collagen type I (Chemicon). All cultures were then incubated for an additional hour with the appropriate fluorescein-conjugated secondary antibodies (Sigma). Nuclei were counterstained with propidium iodide (Sigma). Secondary antibodies alone were also used as an additional control. All cultures were examined with a Nikon Eclipse E1000 microscope attached to a cooled charge-coupled device camera (Retiga EX; QImaging, Surrey, BC, Canada). All immunodetected ECM components were quantitatively analyzed with the computer-generated video analysis system (Image-Pro Plus software; Media Cybernetics, Silver Spring, MD, USA). In each group, 20 low-power fields (×20) from three separate cultures were analyzed, and the areas occupied by the particular immunodetectable components were
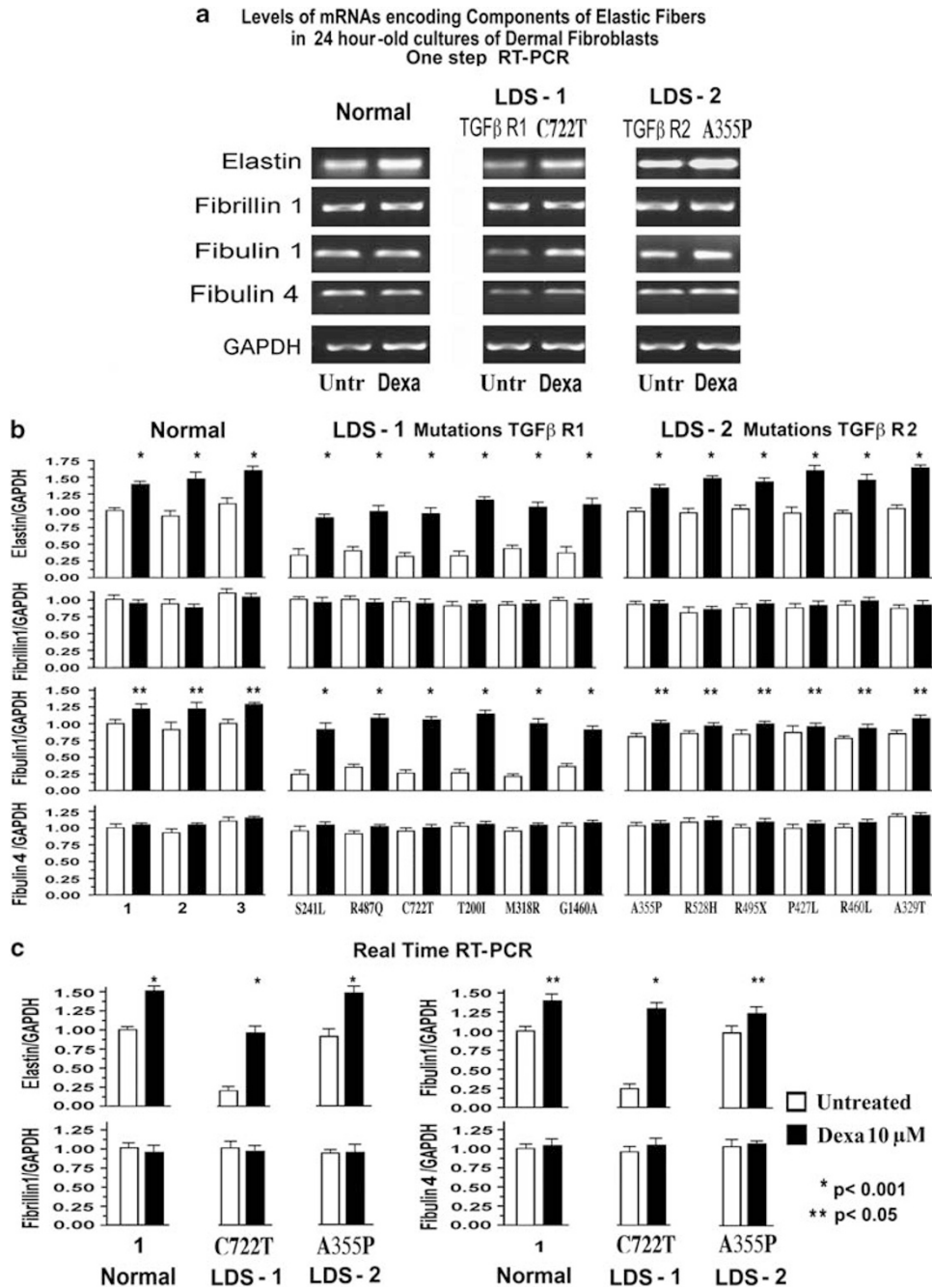

Figure 1 Representative images of RT-PCR analysis (**a**) and quantification of one-step RT-PCR evaluation of mRNA levels encoding components of elastic fibers in 24-h-old cultures of normal fibroblasts and fibroblasts derived from 12 patients with LDS (**b**). The 24-h-long treatment with 10 μM dexamethasone (Dexa) significantly upregulated the abnormally low levels of mRNAs encoding tropoelastin and fibulin 1 observed in untreated cultures of fibroblasts derived from patients with LDS-1 and further enhanced the basic levels of mRNAs encoding these components of elastic fibers in normal and LDS-2 fibroblasts. Treatment with dexamethasone did not induce any increase in the steady-state levels of mRNAs encoding fibrillin 1 and fibulin 4. (**c**) Results of the real-time RT-PCR estimation of mRNAs encoding ECM components, performed in representative cultures of normal fibroblasts and fibroblasts derived from patients with LDS 1 and LDS 2, were proportionally similar to results obtained by the one-step RT-PCR.
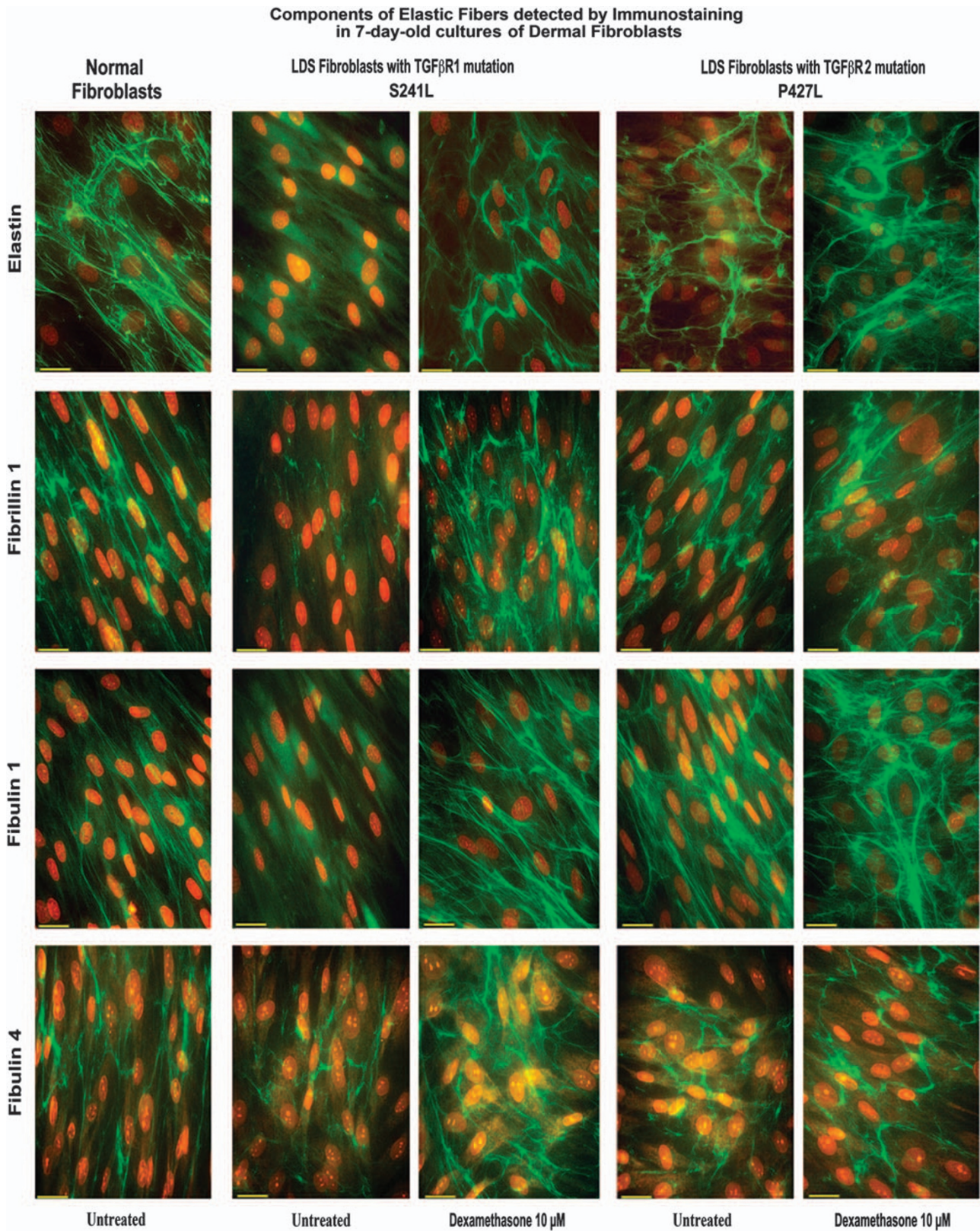
Figure 2 Representative micrographs depicting 7-day-old cultures of normal skin fibroblasts and LDS fibroblasts immunostained with antibodies specific to indicated components of elastic fibers (scale bars=10 µm). The LDS-1 fibroblasts bearing TGFβ-R1 gene mutations deposited only negligible amounts of immunodetectable elastin, fibrillin 1, fibulin 1 and fibulin 4 when compared with normal fibroblasts and LDS-2 fibroblasts with TGFβ-R1 gene mutations. All dexamethasone-treated cultures of LDS fibroblasts proportionally improved their net deposition of all tested proteins and assured their assembly into thick elastic fibers.

quantified. In cultures probed with antibody-recognized collagen type I, we separately estimated the green fluorescence present inside and between cells. Then, the percentage of fluorescence detected in both compartments was estimated, relatively to the total fluorescence detected in the given analyzed field.

Electron microscopy

Four randomly selected 5-day-old cultures of LDS fibroblasts that were maintained in the presence and absence of 10 μM dexamethasone were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer containing 2.5% tannic acid, postfixed with 1% osmium tetroxide in the same buffer, dehydrated in ethanol and embedded in Epon. This preparation gave high contrast of elastin when thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Dermal fibroblasts derived from LDS patients displayed abnormal pattern of expression of genes encoding elastin and fibulin 1

Initial RT-PCR-based comparison of primary cultures of dermal fibroblasts revealed that fibroblasts derived from six LDS patients with different TGFβ-R1 mutations consistently demonstrated an average $72 \pm 6\%$ less tropoelastin-encoding mRNA and $87 \pm 6\%$ less fibulin 1-encoding mRNA than normal fibroblasts (Figure 1). In contrast, fibroblasts from six LDS patients with mutations of the TGFβ-R2 gene showed normal levels of tropoelastin mRNA and only $9 \pm 4\%$ less fibulin 1-encoding mRNA than normal fibroblasts. All tested LDS fibroblasts produced near-normal levels of fibrillin 1- and fibulin 4-encoding mRNAs.

Dexamethasone stimulated elastin and fibulin 1 gene expression

RT-PCR analysis showed that treatment of fibroblasts with 10 μM dexamethasone for 24 h returned the initially low levels of elastin and fibulin 1 mRNAs to near-normal levels in all LDS fibroblasts with TGFβ-R1 gene mutations. Dexamethasone also enhanced the transcription of mRNAs for elastin and fibulin 1 in normal fibroblasts and in LDS fibroblasts with mutations of TGFβ-R2 (Figure 1). Treatment with dexamethasone did not induce any increase in the steady-state levels of mRNAs encoding fibrillin 1 and fibulin 4. In contrast, all fibroblasts treated with dexamethasone demonstrated a slight decrease in the levels of mRNA encoding collagen 1 (Figure 4a). Importantly, the results of the additional real-time RT-PCR estimation of mRNAs encoding ECM components, performed in the representative cultures of normal fibroblasts and fibroblasts derived from patients with LDS 1 and LDS 2, were proportionally similar to results obtained by the one-step RT-PCR (Figure 1c).

Dexamethasone induced remarkable improvement in the net deposition of normal elastic fibers and enhanced secretion of collagen 1 in cultures of LDS fibroblasts

Immunohistochemical staining of parallel 7-day-old cultures of skin fibroblasts with anti-elastin, anti-fibrillin 1, anti-fibulin 1 and anti-fibulin 4 antibodies (Figure 2), followed by morphometric quantitative analysis (Figure 3), showed that LDS fibroblasts with TGFβ-R1 gene mutations deposited only negligible amounts of immunodetectable elastin, fibrillin 1 and fibulin 1 that would be organized into elastic fibers compared with normal fibroblasts. Only fibulin 4 was deposited in the fibrillar form, but the detectable amount was also lower than in cultures of normal fibroblasts. Deposition of all immunodetected elastic fiber components was markedly higher in cultures of fibroblasts with TGFβ-R2 mutations than those with TGFβ-R1 mutations and only slightly lower than those detected in parallel cultures of normal fibroblasts. The results of morphometric evaluation also indicated that fibroblasts with TGFβ-R2 gene

mutations demonstrated abnormal intracellular retention of immuno-detectable collagen 1, coexistent with a significantly lower number of extracellular collagen fibers than could be detected in cultures of normal fibroblasts or cells with mutations in TGFβ-R1 gene (Figure 4b). All dexamethasone-treated LDS fibroblasts improved their net deposition of all tested components of elastic fibers that organize into normal elastic fibers. The improvement in the elastic fiber deposition following treatment with dexamethasone was particularly striking in cultures of fibroblasts with TGFβ-R1 gene mutations. These data correlated well with results of a quantitative assay measuring deposition of [^{3}H]-valine-labeled insoluble elastin (Figure 3b).

Dexamethasone treatment also improved the net deposition of extracellular collagen fibers in all cultures of LDS fibroblasts. This effect was particularly impressive in cultures of cells with TGFβ-R2 gene mutations, where dexamethasone rectified the intracellular retention of collagen type I and enhanced a concomitant deposition of collagen fibers outside of the cell in the extracellular matrix. EM confirmed the findings obtained using light microscopy immuno-histochemistry (Figure 5).

DISCUSSION

TGFβ1 and TGFβ2 are multipotential cytokines involved in the development of numerous body organs and systems, including craniofacial structures and the cardiovascular system. They interact with different receptors coded by the TGFβ-R1 and TGFβ-R2 genes. The ligand TGFβ1 binds first to a homodimer of the TGFβ-R2 subunits and then recruits a homodimer of the TGFβ-R1 protein, consecutively activating it by multiple phosphorylation of its glycine-serine-rich region.[14] In turn, the activated TGFβ-R1 protein propagates downstream signals causing phosphorylation of the Smad family members, the known regulators of transcription of multiple genes. Despite numerous reports of different mutations in the TGFβ receptor genes and the variability of clinical manifestations associated with LDS, little is known about the cellular mechanisms responsible for development of particular phenotypic features. The existence of significant phenotypic overlap between LDS and MFS suggests that the causative mechanism may be similar in both conditions. Para-doxically increased TGFβ signaling has a significant role in MFS and it has been suggested that increased TGFβ signaling also contributes to other MFS-like disorders, including LDS. Increased TGFβ signaling is thought to be triggered by the absence of fully assembled fibrillin 1, a protein that normally sequesters TGFβ, thereby contributing to downregulation of its functional potential. There is solid evidence that the genomic fibrillin 1 deficiency in MFS results in end-organ damage via this route.[9,15] It has been also documented that TGFβ signaling can be blocked by losartan, an angiotensin II type I receptor antagonist, resulting in rescue of aortic dilatation in the Marfan transgenic mouse model, leading to a randomized controlled trial of losartan in human MFS patients.[16,17] It has been also suggested that mutations in TGFβ receptor-encoding genes (TGFβ-R1 and 2) would lead to persistent and increased TGFβ-induced signals in LDS patients.[1] However, this suggestion does not correlate well with the fact that TGFβ can normally induce transcription of elastin gene and enhance elastin mRNA stability, as well as induce transcription genes encoding lysyl oxidases (LOXes) that are responsible for crosslinking of both elastin and collagen precursors.[18–20] It is not clear yet whether the initially impaired fetal elastogenesis and aberrant collagen deposition directly contribute to early aortic dissections in children with LDS.[11] However, the result of our study provides a novel insight into the pathomechanism of these features. We have demonstrated a significant reduction
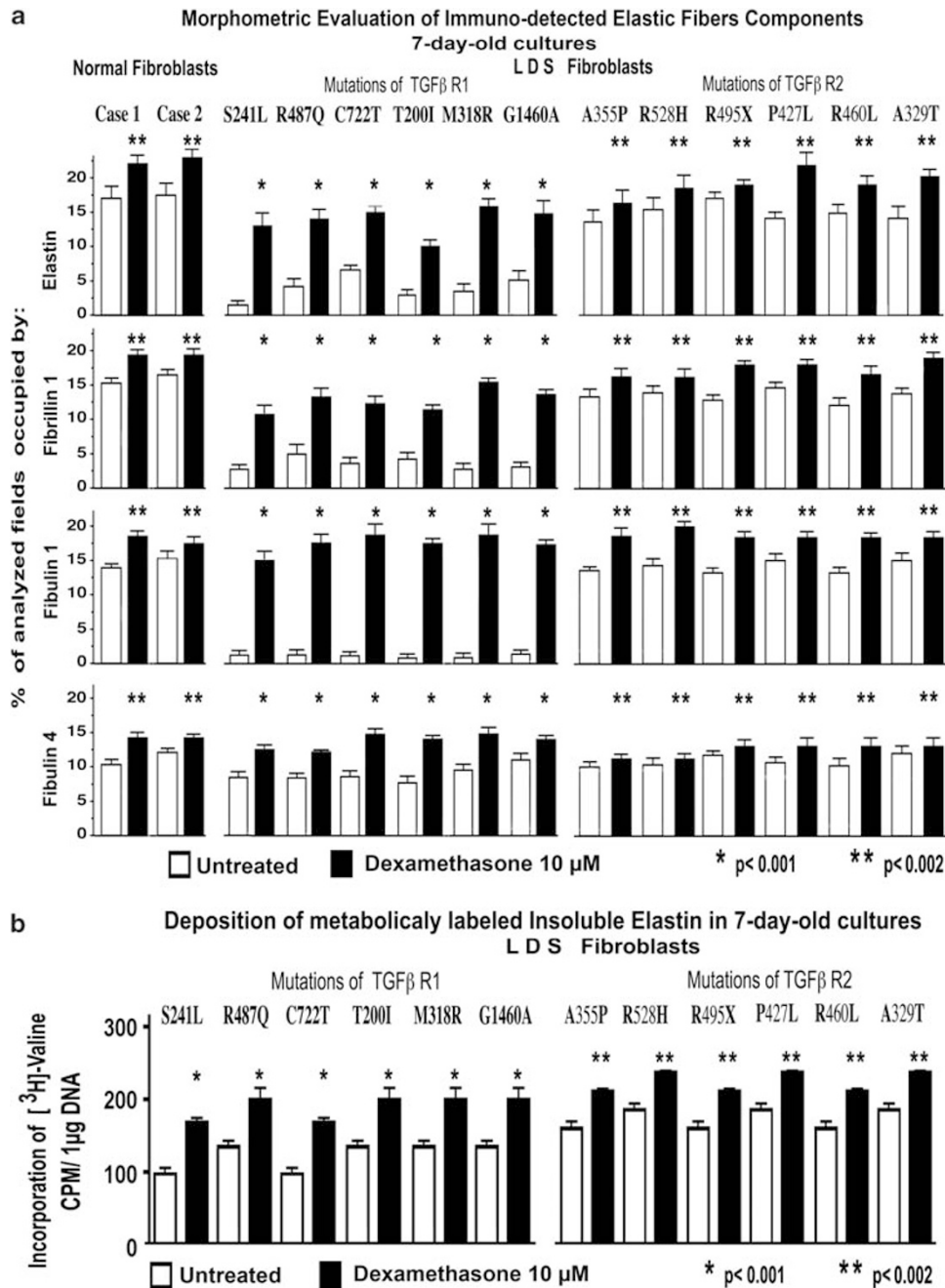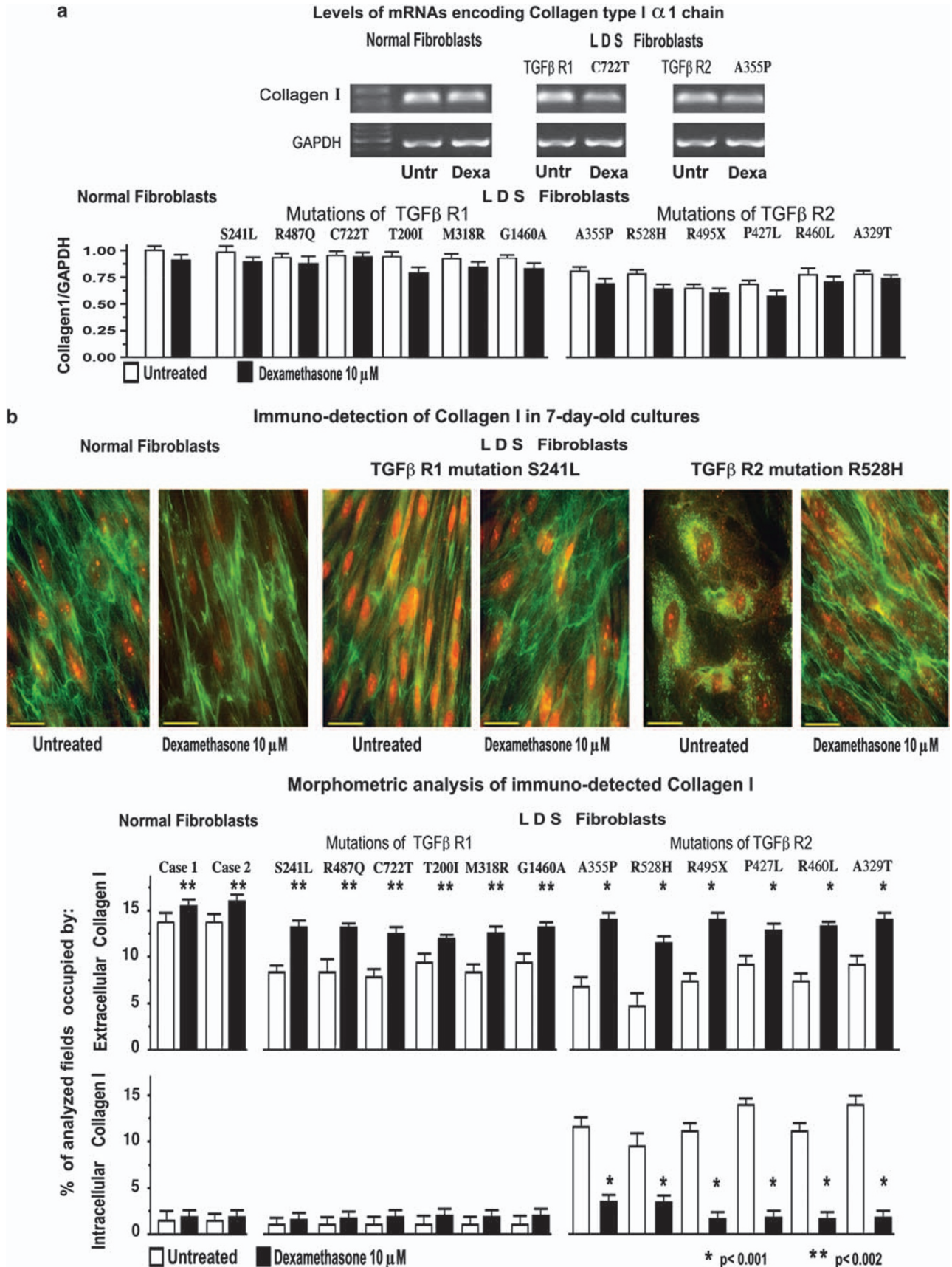
Figure 3 (a) Results of the morphometric quantitative analysis confirmed that levels of net deposition of immunodetected elastin, fibrillin 1, fibulin 1 and fibulin 4 in untreated 7-day-old cultures of LDS-1 fibroblasts with six different mutations of TGFβ-R1 were consistently and significantly lower than those observed in the cultures of normal and LDS-2 fibroblasts. The results also confirm that all dexamethasone-treated cultures proportionally improved their net deposition of all four elastic fiber components. (b) Results of a quantitative assay measuring incorporation of [^{3}H]-valine into the insoluble elastin demonstrate that cultured LDS fibroblasts carrying mutations of both TGFβ receptor-encoding genes produced more insoluble elastin in response to dexamethasone treatment.

in the deposition of elastic fibers in cultured LDS fibroblasts with TGFβR1 gene mutations and faulty deposition of collagen fibers in the cultures of LDS fibroblasts with TGFβ-R2 gene mutations. Most importantly, for the first time we have established that treatment with dexamethasone leads to a remarkable reversal of these pathological features observed in both types of LDS fibroblasts. Although the detailed analysis of all TGFβ signaling pathways was beyond the scope

of the present study, we may only speculate that the reported abnormal deposition of extracellular matrix components occur in result of either an inadequate or an imbalanced transduction of TGFβ signaling in analyzed LDS fibroblasts bearing mutated TGFβ receptors. We have shown consistently lower than normal expression of elastin and fibulin 1 genes in fibroblasts derived from six LDS patients with TGFβR1 mutations. Despite the fact that all tested LDS fibroblasts
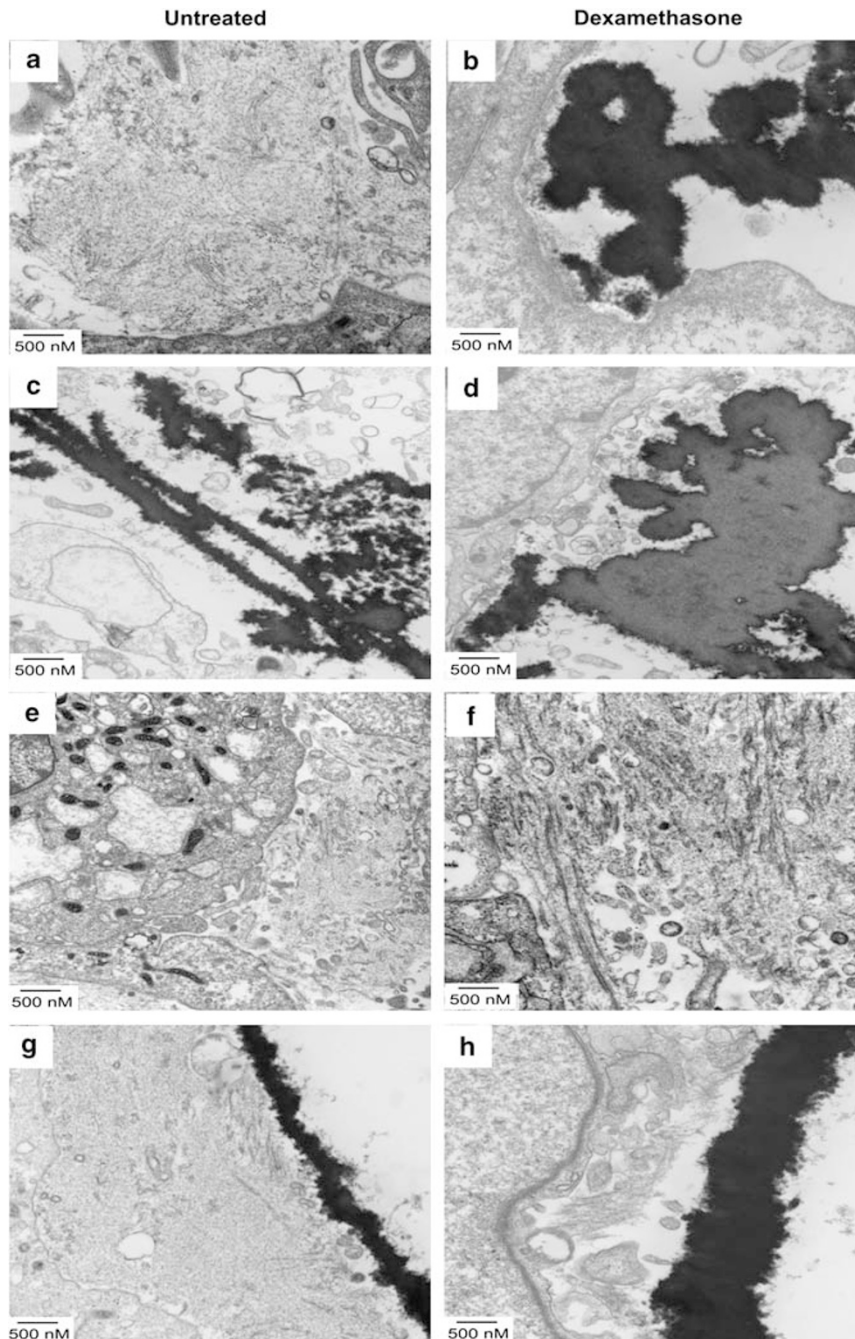
a Levels of mRNAs encoding Collagen type I α1 chain

b Immuno-detection of Collagen I in 7-day-old cultures

Morphometric analysis of immuno-detected Collagen I

* p< 0.001 ** p< 0.002

Figure 5 The representative EM micrographs demonstrate that the matrix produced by cultured LDS fibroblasts with TGFβ-R1 gene mutation consists of tightly packed collagen fibers and a striking lack of elastic fibers (**a**) and that dexamethasone treatment induced restoration of elastic fibers (**b**). Fibroblasts bearing mutation of TGFβ-R2 gene that produced thin and immature elastic fibers, when cultured in normal medium (**c**), demonstrated thick and well-organized elastic fibers, following dexamethasone treatment (**d**). In contrast to untreated LDS fibroblasts with TGFβ-R2 mutation that show a storage of fibrous collagen material in intracellular vacuoles and only single thin collagen fibers in ECM (**e**), the cultures treated with dexamethasone demonstrate normal collagen fibers in the extracellular space (**f**). Cultures of normal fibroblasts treated with dexamethasone (**h**) also produce more extracellular collagen and thicker elastic fibers than their untreated counterparts (**g**).

Figure 4 (**a**) Images of the representative RT-PCR analysis (upper panel) and results of a quantitative RT-PCR evaluation (lower panel) demonstrate that fibroblasts derived from all 12 patients with LDS show almost similar steady-state levels of mRNAs encoding collagen type I α1 chain as parallel cultures of normal fibroblasts, and that 24-h-long treatment with dexamethasone (Dexa) leads to a slight decrease in the levels of mRNA encoding collagen type 1. (**b**) Representative micrographs depicting cultures immunostained with anti-collagen type I antibody (upper panel) (scale bars$=$20 μm) and results of morphometric evaluation of all immunostained cultures (lower panel) indicate that fibroblasts with TGFβ-R2 gene mutations show an abnormal intracellular retention of immunodetectable collagen type I, coexisting with a significantly lower number of extracellular collagen fibers that could be detected in cultures of normal fibroblasts or fibroblasts bearing TGFβ-R1 gene mutations. Dexamethasone treatment rectified the abnormal intracellular retention of collagen I in fibroblasts with TGFβ-R2 mutations and improved the net deposition of extracellular collagen fibers in all cultures of tested LDS fibroblasts.

with TGFβR1 mutations revealed normal levels of the steady-state expression of fibrillin 1 and fibulin 4 genes, they ultimately demonstrated a marked decrease in deposition of immunodetected microfibrils. This coincided with the conspicuously low extracellular deposition of insoluble (crosslinked) elastin and lack of normal assembly of immunodetected elastic fibers. The possible involvement of aberrant TGFβ signaling, potentially interfering with the net production of microfibrils, is not entirely clear at this point. However, our data strongly indicate that the presence of mutations in TGFβ-R1, but not mutations in TGFβ-R2, effectively interferes with the propagation of the elastogenic signals normally induced by TGFβ.

Up to eight different transcription start sites have been identified in the elastin gene, indicating that the transcription of this gene is controlled by complex mechanisms. Numerous factors and hormones such as TNFα1, insulin-like growth factor I, interleukin-1β and interleukin 10 have all been shown to induce signals that eventually affect tropoelastin synthesis at the promoter region, after first interacting with their respective cell surface receptors.[21] Glucocorticoids, however, can effectively by-pass cell membrane receptors and interact directly with elastin gene regulatory elements to produce upregulation of the elastin gene transcription.[12] It has been specifically demonstrated that glucocorticoid-induced signaling can use alternative pathways and exert its effect without involvement of TGF-β-dependent signals.[22] With this in mind, we suggest that dexamethasone, through a direct interaction with the promoter region of the elastin gene, might serve as an alternative elastogenic stimulator for LDS fibroblasts bearing mutated TGFβ-R1 that are apparently unable to properly propagate the TGFβ-induced elastogenic signals.[23] At the same time, we documented that treatment with dexamethasone also induced a consistent increase in deposition of major components of microfibrillar scaffold of elastic fibers, fibulin 1 and fibrillin 1. Although the recovery of fibulin 1 microfibrils (Figure 2) can be linked to the observed (Figure 1) and previously documented[24] primary stimulation of fibulin 1 gene expression by dexamethasone, the subsequent remarkable increase in the deposition of fibrillin 1 microfibrils has not been preceded by any increase in fibrillin 1 mRNA level. As the basic expression of fibrillin 1 was not substantially harmed in LDS fibroblasts bearing mutated TGFβ-R1, we may additionally speculate that dexamethasone would also alleviate an aberrant signaling contributing to under-expression or inactivation of furin. This protein convertase is normally responsible for the post-translational processing of pro-fibrillin 1 molecules, the prerequisite for their homo-polymerization and assembly into the microfibrillar scaffold of elastic fibers.[25,26]

Interestingly, the apparent aberration in TGFβ signaling in LDS fibroblasts did not affect the expression of collagen 1. However, we found that all six fibroblasts with TGFβ-R2 mutations demonstrated lower than normal deposition of extracellular collagen fibers and a peculiar intracellular accumulation of fibrous material detectable with antibody to collagen type I. This observation suggests that abnormal TGFβ-generated signaling may result in either inhibition of the proper intracellular processing and secretion of collagen I precursors, or with the enhancement of phagocytosis of the already secreted tropocollagen molecules, which could not be properly crosslinked in the extracellular space. The latter possibility seems to be more probable as the LDS fibroblasts demonstrated lower than normal expression of LOX and a similar intracellular accumulation of collagen 3 has been described as a cardinal feature in EDS vascular type, characterized by LOX deficiency.[27]

Currently, we are unable to exclude the involvement of other parallel mechanisms that could be triggered by dexamethasone, and

further studies are needed for the detailed elucidation of the phenomenon observed in our in vitro studies. It would be particularly rational to learn, for example, whether dexamethasone would also upregulate expression of the non-mutated alleles encoding normal TGFβ receptors in the heterozygotic LDS patients.

In summary, we show that alterations of normal elastogenesis and collagen I deposition heavily contribute to the LDS phenotypes. Most importantly, observation of the remarkable improvement in deposition of both fibrillar ECM components following treatment with dexamethasone raises the intriguing question of whether prenatal treatment with this drug may lead to amelioration of the LDS phenotypes. Prenatal dexamethasone treatment may have practical utility because LDS can be diagnosed prenatally[28] and fetal LDS patients may be identified via their affected parent. The possibility of prenatal treatment is particularly important in light of the fact that organogenesis-relevant elastogenesis occurs predominantly in the last trimester of human fetal life and in the early weeks of postnatal life.[21,29] This provides a potential window of opportunity for prenatal treatment with dexamethasone in cases at risk or known to be affected with LDS. The observation that dexamethasone ameliorated intracellular storage of collagen type I and normalized deposition of collagen fibers in fibroblasts with mutations in the TGFβ-R2 gene additionally supports our suggestion that early prenatal treatment with dexamethasone may alleviate connective tissue problems observed in LDS patients.

Dexamethasone has been already safely and effectively used prenatally to suppress adrenal gland activity in cases with congenital adrenal hyperplasia in female fetuses.[30] The safety of pulse-dose corticosteroids has been established in pregnancy through their widespread use over the past 15 years in the prevention of prematurity-related respiratory distress syndrome and associated morbidities.[31,32] Furthermore, short-term treatment of LDS patients before vascular surgery may improve the strength of aortic wall, diminish the risk of aneurysm rupture and potentially increase the successful outcome of the surgical procedure.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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