Fluctuations of Intracellular Iron Modulate Elastin Production*

Severa Bunda, Nilo Kaviani, and Aleksander Hinek‡

From the Cardiovascular Research Program, The Hospital for Sick Children and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5G 1X8, Canada

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Production of insoluble elastin, the major component of elastic fibers, can be modulated by numerous intrinsic and exogenous factors. Because patients with hemolytic disorders characterized with fluctuations in iron concentration demonstrate defective elastic fibers, we speculated that iron might also modulate elastogenesis. In the present report we demonstrate that treatment of cultured human skin fibroblasts with low concentration of iron 2–20 μM (ferric ammonium citrate) induced a significant increase in the synthesis of tropoelastin and deposition of insoluble elastin. Northern blot and real-time reverse transcription-PCR analysis revealed that treatment with 20 μM iron led to an increase of ~3-fold in elastin mRNA levels. Because treatment with an intracellular iron chelator, desferrioxamine, caused a significant decrease in elastin mRNA level and consequent inhibition of elastin deposition, we conclude that iron facilitates elastin gene expression. Our experimental evidence also demonstrates the existence of an opposite effect, in which higher, but not cytotoxic concentrations of iron (100–400 μM) induced the production of intracellular reactive oxygen species that coincided with a significant decrease in elastin message stability and the disappearance of iron-dependent stimulatory effect on elastogenesis. This stimulatory elastogenic effect was reversed, however, in cultures simultaneously treated with high iron concentration (200 μM) and the intracellular hydroxyl radical scavenger, dimethylthiourea. Thus, presented data, for the first time, demonstrate the existence of two opposite iron-dependent mechanisms that may affect the steady state of elastin message. We speculate that extreme fluctuations in intracellular iron levels result in impaired elastic fiber production as observed in hemolytic diseases.

Mature elastic fibers and laminae provide extensibility and elastic recoil to vascular walls and ligaments and form a connective tissue framework of lungs, elastic cartilage, and skin (1, 2). They are complex structures made of polymeric (insoluble) elastin and 12-nm microfibrils that consist of several glycoproteins, e.g. fibrillins, fibulins, and microfibril-associated glycoproteins (3–6). Elastic fiber formation (elastogenesis) is a complex process involving several intracellular and extracellular events. Cells (fibroblasts, endothelial cells, chondroblasts, or vascular smooth muscle cells) must first synthesize and secrete numerous glycoproteins to form a microfibrillar scaffold upon which tropoelastin, the precursor peptides, are properly assembled and covalently cross-linked by lysyl oxidase into a resilient polymer, insoluble elastin (7–10). Production of elastin reaches its highest levels in the third trimester of the fetal life and steadily decreases during early postnatal development (11, 12). In undisturbed tissues, elastic fibers may last over the entire human lifespan (13, 14).

The net deposition of elastin appears to be controlled on both the transcriptional level (tropoelastin mRNA message expression (15, 16)) and post-transcriptional level (tropoelastin message stability (17–19)). There are also several other post-transcriptional events, which control secretion of tropoelastin monomers and their proper extracellular assembly (20, 21) and regulate the cross-linking of tropoelastin into the polymeric “insoluble” elastin, the most durable element of the extracellular matrix (14).

In addition to primary elastinopathies that have been directly linked to alterations in the elastin gene (supravalvular aortic stenosis, Williams-Beuren syndrome, and cutis laxa (22–24)), a number of secondary elastinopathies have been described, caused by functional imbalance of other structural and auxiliary factors regulating elastic fiber deposition (Marfan disease, GM-1-gangliosidosis, Morquio B, Hurler disease, Costello syndrome, Ehlers Danlos syndrome, pseudoxanthoma elasticum (10, 25, 26, 27–29)).

Diffuse elastic fiber defects, resembling those reported in inherited pseudoxanthoma elasticum, have also been detected in patients with β-thalassaemia and sickle cell anemia (30), and in other hemolytic anemias (31, 32). The genetic basis for these diseases cannot be directly linked to any structural or regulatory components involved in elastic fiber production (30, 33, 34). However, it has been suggested that the accumulation of iron in these patients, resulting from hemolysis, increased iron absorption, and multiple blood transfusions may lead to acquired elastic tissue defects (30).

Iron is a physiologically essential nutritional element for all life forms (35). It plays critical roles in electron transport and cellular respiration, oxygen transport by hemoglobin, cell proliferation, and differentiation (36). It has been shown that modulating intracellular iron levels may also affect expression of numerous genes that are not directly involved in iron metabolism, such as protein kinase C-β, an important component of intracellular signaling pathways (37), or those encoding extracellular matrix (ECM) components (36, 38). It has been demonstrated that dietary iron overload in rats resulted in an

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‡ To whom correspondence should be addressed: Cardiovascular Research Program, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario M5G 1X8, Canada. Tel.: 416-813-5918; Fax: 416-813-7480; E-mail: alek.hinek@sickkids.on.ca.

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increase in the steady-state level of pro-α2(I)-collagen in hepatoctyes (39) and that 50 μM iron treatment stimulated collagen gene expression in cultured stromal hepatic cells by inducing the synthesis and binding of Sp1 and Sp3 transcription factors to two regulatory elements located in the collagen α1 (I) promoter region (40). On the other hand, iron loading in cultured cardiac myocytes and fibroblasts decreased the expression of transforming growth factor-β, biglycan, and collagen type I mRNA, whereas it facilitated the expression of decorin mRNA.

Interestingly, iron deprivation exerted a similar effect, suggesting that the expression of these genes involved in extracellular matrix production is regulated by certain iron-dependent mechanisms (41).

The molecular basis of iron-dependent mechanism(s) regulating the expression of ECM-encoding genes is not well understood. Because raising levels of iron may overwhelm the iron binding capacity of transferrin, resulting in the appearance of non-transferrin bound iron (42), which is capable of catalyzing the formation of the hydroxyl radicals (through the Fenton and Haber-Weiss reactions) (43), it has been suggested that iron-dependent induction of reactive oxygen species (ROS) may modulate the transcription of these genes (36, 44). The possibility of iron-dependent oxidative damage to elastic fibers has also been suggested, but not proven (30).

The present study was designed to elucidate the mechanisms by which iron might modulate elastogenesis. We utilized normal human skin fibroblasts that are capable of elastic fiber production in vitro to explore whether changes in concentrations of iron would affect elastin gene expression and the subsequent deposition of elastic fibers. We also tested the influence of different iron concentrations on generation of intracellular ROS, and attempted to identify on which level these iron-induced ROS affect elastogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials—** All chemical-grade reagents, catalase, desferrioxamine (DFO), dichlorobenzimidazole riboside (DBR), dimethylthiourea (DMTU), ferric ammonium citrate (FAC), superoxide dismutase, and tempol were all obtained from Sigma, and Dulbecco’s modified eagle’s medium, fetal bovine serum (FBS), 0.2% trypsine-0.02% EDTA, and other cell culture products from Invitrogen. We obtained 5-(and-6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate and acetyl ester (CM-H2DCFDA) from Molecular Probes (Eugene, OR). Polyclonal antibody to tropoelastin was purchased from Elastin Products (Owensville, MO).

Secondary antibody fluorescein isothiocyanate-conjugated goat anti-rabbit was purchased from Sigma. DNeasy Tissue system for DNA assay and RNasey Mini Kit for isolation of total RNA were purchased from Qiagen (Mississauga, Ontario, Canada). A OneStep RT-PCR kit was purchased from Qiagen. A SuperScript First-Strand Synthesis System for RT-PCR was purchased from Invitrogen. TaqMan Universal PCR master mix, TaqMan GAPDH control, and Assays-on-Demand Gene Expression probe for elastin were purchased from Applied Biosystems (Foster City, CA). The radiolabeled reagents, [3H]valine and [3H]thymidine, and Rediprime (II) Random Primer labeling system were purchased from Amersham Biosciences. Hybridization solution Miracle Hyb was purchased from Stratagene (Cedar Creek, TX), and the human GAPDH control was purchased from Clontech (Palo Alto, CA).

**Cultures of Normal Human Skin Fibroblasts—** Fibroblasts grown from skin biopsy explants of six normal subjects, aged from 2 months to 10 years, were obtained from the cell repository at The Hospital for Sick Children in Toronto with the permission of the Institutional Ethics Committee. Fibroblasts were routinely passaged by trypsinization and maintained in Dulbecco’s modified eagle’s medium supplemented with 1% antibiotics/antimycotics, and 10% FBS. In all described experiments passages 2–6 were used.

In experiments aimed at assessing ECM production, fibroblasts were initially plated (100,000 cells/dish) and maintained in normal medium until confluency at which point they produce abundant ECM. Confluent cultures were then treated for 72 h with or without FAC producing iron concentrations from 2 to 200 μM. The low iron concentration (2 and 20 μM) of iron utilized in the present study remained in the range that did not induce any disturbances in cellular metabolism when tested by other investigators (45). The high iron concentration (200 μM) was comparable to concentrations used in studies of iron overload (46, 48).

In some experiments the membrane permeable ferric iron chelator, DFO, was added 30 min prior to FAC treatment. For the experiments conducted in the presence of various antioxidants, the antioxidants were applied 1 h prior to FAC treatment. For experiments conducted in serum-free conditions, 7-day-old confluent fibroblast cultures were starved for 12 h in serum-free medium and incubated with various concentrations of iron (as FAC) for additional 72 h in serum-free medium.

**Assessment of Intracellular ROS Levels—** The ROS-sensitive fluorescent probe, CM-H2DCFDA for three additional hours. The cells were transfected to the serum-free medium for synchronization of their cell cycle and then maintained in the presence or absence of FAC (2–200 μM) for three additional hours. The cells were then washed in PBS and treated with cold 5% trichloroacetic acid twice incubated at room temperature for 30 min, and 200-μl aliquots of each culture mixed with scintillation fluid and counted (21). Aliquots taken from each culture were also used for DNA determination according to Ref. 47, using the DNeasy Tissue System from Qiagen. Final results reflecting amounts of metabolically labeled insoluble elastin in individual cultures were normalized per their DNA content and expressed as cpm/μg of DNA. In separate experiments, the specified treatment described in the figure legends was added along with 2 μCi of [3H]valine/ml media to confluent cultures of normal human skin fibroblasts in 35-mm culture dishes (100,000 cells/dish) in quadruplicates for 72 h. The conditioned media was then removed, the cell layers were washed, and incorporation of [3H]valine into the insoluble elastin was assessed as described above.

**Assessment of Cell Proliferation—** Normal human skin fibroblasts were suspended in Dulbecco’s modified eagle’s medium containing 10% FBS and plated in 35-mm culture dishes (100,000 cells/dish) in quadruplicates. Twenty-four hours later, the cells were transferred to the serum-free medium for synchronization of their cell cycle and then maintained in the presence or absence of FAC (2–200 μM) and 2 μCi of [3H]thymidine/ml media with 10% FBS for 72 h. These cultures were then washed in PBS and treated with cold 5% trichloroacetic acid twice for 10 min at 4 °C. One-half milliliter of 0.3 N NaOH was added to all dishes, incubated at room temperature for 30 min, and 200-μl aliquots of each culture mixed with scintillation fluid and counted (21).

**Assays of Intracellular ROS Levels—** The ROS-sensitive fluorescent probe, CM-H2DCFDA has been used to detect oxidative activity in cultured fibroblasts (48). This probe passively diffuses into the cell interior, and only upon oxidation is a fluorescent product released that can be visualized under fluorescent microscope or captured by flow cytometry. FAC was added at 480 nm. The intracellular ROS production, normal human skin fibroblasts were plated on glass coverslips in 35-mm dishes (50,000 cells/dish) and grown to confluency. The cells were then washed with PBS and incubated with or without 10 μM CM-H2DCFDA for 30 min in fresh media. The cells were then washed again in PBS and incubated with new media in the presence or absence of FAC (2–400 μM) for three additional hours. The cells were then washed twice with PBS before being mounted to the glass slides,
and the images were captured using a fluorescence microscope under identical parameters of contrast and brightness.

In addition, the quantification of this reaction was performed by flow cytometry (λ excitation, 480 nm; λ emission, 520 nm). Quadruplicate cultures of fibroblasts were preincubated with CM-H$_2$DCFDA and maintained in the presence or absence of FAC as described above. To reduce stress-induced oxidant activation, the cells were cooled and harvested by trypsinization at 4 °C. They were then collected by centrifugation (4 °C, 1000 rpm for 3 min), washed in cold PBS, and fixed with 4% formaldehyde for 10 min in the dark and analyzed by flow cytometry (FACSCalibur, BD Biosciences).

Northern Blots—Normal human skin fibroblasts were grown to confluence in 100-mm culture dishes. Fresh media was added along with or without 2, 20, and 200 μM of FAC for 24 h. Total RNA was isolated using RNeasy Mini Kit according to manufacturer’s instructions, and 10 μg were resolved by electrophoresis on formaldehyde-1% agarose gels. Recovery of 18 S and 28 S rRNA was analyzed using ethidium-bromide staining and image analysis on a Gel Doc 1000 optical-system (Bio-Rad). RNA was transferred onto Hybond-N membrane (Amersham Pharmacia Biotech) by capillary transfer in 10× SSC and immobilized by UV cross-linking. Human elastin cDNA recombinant probe H-11 (49, 50) was radiolabeled with $^{32}$P random primer method and incubated
FIG. 2. The influence of iron on tropoelastin production, mRNA levels, and message stability. A, results of quantitative assay of newly produced, metabolically labeled, and immunoprecipitable soluble tropoelastin. Cultures treated for 72 h with 2 and 20 μM iron (FAC) synthesized more [3H]valine-labeled tropoelastin than untreated counterparts (*, p < 0.05). Cultures treated with higher iron concentrations (100 and 200 μM) demonstrated lower tropoelastin production as compared with those treated with 20 μM iron (**, p < 0.05). B, Northern blots and TaqMan real-time PCR analysis demonstrate that 24-h exposure of fibroblasts to 2 and 20 μM of iron (FAC) cause a significant increase in elastin mRNA steady state levels as compared with untreated control (*, p < 0.05). Both analyses demonstrate, however, that 24-h treatment with 200 μM iron significantly reduces tropoelastin mRNA levels, as compared with cultures treated with 20 μM iron (**, p < 0.05). The intensity of elastin message signal detected by Northern blotting was assessed by densitometry after normalization to GAPDH message levels, and the corresponding values are shown in the bar graph in arbitrary units. Elastin mRNA levels assessed by TaqMan real-time PCR analysis were normalized to the corresponding levels of GAPDH mRNA and expressed as a percentage of untreated control values. C, the influence of iron on elastin mRNA stability in normal
overnight at 42 °C with the membrane in Miracle Hyb solution at a concentration of 2.5–5 × 10^6 cpm/ml. The membrane was washed to high stringency, and the bound radioactivity was visualized by autoradiography and quantified by scanning densitometry (Gel Doc 1000). RNA loading and transfer were evaluated by probing with a glycolaldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to which relative elastin mRNA values were normalized.

Quantitative TaqMan RT-PCR—To confirm the expression level of elastin mRNA in the presence of 2, 20, and 200 μM FAC obtained by Northern blot analysis, we also conducted quantitative RT-PCR. To assess the effect of iron on elastin mRNA stability, parallel quadruplicate cultures were grown to confluency in 100-mm dishes. Media were then changed, supplemented with 60 μM transcription blocker, DRB (51), and cultures were maintained in the presence or absence of 20 and 200 μM FAC for 0, 6, 12, and 24 h. Total RNA was extracted using the RNaseasy Mini kit, according to manufacturer’s instructions, at indicated time points. The mixtures were subjected to reaction, which was performed in 12 μl total RNA, oligo(dT)6s, and the SuperScript First-Strand synthesis system (Invitrogen) according to the manufacturer’s instructions.

Elastin mRNA levels were measured by real-time quantitative PCR method performed on the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). For each treatment two distinct amplifications were carried out in parallel to amplify elastin cDNA and GAPDH cDNA. Amplification reactions were performed in 25-μl volumes containing 30 ng of cDNA per treatment in triplicate, 12.5 μl of 2× Taqman Universal PCR Master Mix (Applied Biosystems), and 1.25 μl of 20× Assays-on-Demand Gene Expression probe for elastin (Applied Biosystems) or TaqMan GAPDH probe (Applied Biosystems). Elastin mRNA levels from each treatment were normalized to the corresponding amount of GAPDH mRNA levels. Water controls and samples with no reverse transcriptase were included to eliminate the possibility of significant DNA contamination. Final results were expressed as the mean of two independent experiments.

One-step RT-PCR—To further confirm the effect of iron on elastin mRNA levels, confluent normal human skin fibroblast cultures were treated with or without intracellular ferric iron chelator, 20 μM DFO in the presence or absence of 20 μM FAC for 24 h. Total RNA was extracted using the RNaseasy Mini kit, according to the manufacturer’s instructions, and 1 μg of total RNA was added to each one-step RT-PCR (Qiagen OneStep 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 elastin and β-actin reactions at 50 °C for 30 min followed by 15 min at 95 °C. The elastin PCR reaction (sense primer: 5'-GGTCAGAAGGATTC-CTATGGT-3', antisense primer: 5'-GGGGCTTGGAGATACCCCAGTG-3') and the β-actin reactions were performed under the following conditions: 25 cycles at 94 °C denaturation for 20 s, 63 °C annealing for 20 s, and 72 °C extension for 1 min, then 1 cycle at 72 °C with a final extension for 10 min.

The β-actin PCR reaction (sense primer: 5'-GGTCAGAAGGATTCCTATGGT-3', antisense primer: 5'-ATTGCCCAATGGTGATGACCTATGTG-3') was designed to produce a 215-bp product. The reactions were performed under the following conditions: 25 cycles at 94 °C denaturation for 20 s, 60 °C annealing for 20 s, and 72 °C extension for 10 min. 5-μl samples of the elastin and β-actin PCR products from each reaction were run on a 2% agarose gel and visualized by silver staining.

Data Analysis—In all biochemical studies quadruplicate samples in each experimental group were assayed in two separate experiments. Mean and standard deviations (S.D.) were calculated for each experimental group, and statistical analyses were carried out by analysis of variance, p value of <0.05 (p < 0.05) was considered significant.

RESULTS

Low and High Doses of Iron Produce Opposite Effects on Production of Insoluble Elastin—We first tested low concentrations of iron 2–20 μM (supplied as FAC) relevant to physiological concentrations of mammalian serum iron (10–30 μM (52)) and then higher concentrations (100 and 200 μM) relevant to iron overload. Immunostaining of confluent fibroblast cultures with anti-elastin antibody revealed that 3-day-long treatment with 2 and 20 μM of iron significantly increased the production of elastic fibers over control levels (Fig. 1A). Interestingly, raising iron concentration to 100 μM did not induce better elastin deposition than treatment with 20 μM, and treatment with 200 μM iron dragged elastin deposition back to the control levels. Metabolic labeling of cultured fibroblasts with [3H]thymidine followed by quantitative assays of insoluble elastin confirmed the results obtained with immunocytochemistry (Fig. 1B). Importantly, the same trend induced by different concentrations of iron was observed in cultures maintained in the presence and absence of serum (Fig. 1B). Results of parallel experiments measuring the incorporation of [3H]thymidine demonstrated that the detected stimulation on elastogenesis in cultures treated with low iron concentrations was not due to increased cellular proliferation rate and that the reverse effect observed at higher concentrations of iron was not due to cellular cytotoxicity (Fig. 1C).

Because the net production of elastic fibers depends on the coordinated expression of multiple factors, we tested the expression of three major factors facilitating elastogenesis by immunofluorescent microscopy after exposure of normal human skin fibroblasts to low (20 μM) and high (200 μM) iron concentrations. In contrast to elastin, the immunodetectable levels of fibrillin-1, a major component of fibrillar scaffold, the elastin-binding protein, required for normal tropoelastin secretion and extracellular assembly, and lysyl oxidase, the enzyme responsible for elastin cross-linking, were not changed in cultures treated with 20 and 200 μM of iron (data not shown).

The Influence of Iron on Elastin mRNA Levels and Message Stability—Because we have shown that incubation of fibroblasts with low (2–20 μM) and high (200 μM) iron concentrations induced opposite effects on the net deposition of insoluble (extracellular) elastin and that 2–200 μM iron concentrations did not stimulate elastolytic activity of serine proteinases (data not shown), we attempted to identify the level on which fluctuations in iron level would affect elastogenesis. Results of following series of experiments demonstrated that low and high iron concentrations induced opposite effects in the neosynthesis of (metabolically labeled) immunoprecipitable tropoelastin that were proportional to the reported changes in the net deposition of insoluble elastin (Fig. 2A). These observations clearly indicated that iron might regulate the earliest stages of elastogenesis, transcription of elastin gene, and/or elastin message stability. Indeed, results of Northern blot hybridization with elastin cDNA probe (corrected for GAPDH mRNA levels) revealed a dose-dependent increase in elastin mRNA levels in cultures incubated for 24 h in the presence of 2 and 20 μM iron. This trend was abolished and returned back to control values in cultures treated with 200 μM of iron (Fig. 2B, left panel). We further examined elastin gene expression under same experimental conditions by quantitative real-time RT-PCR analysis. This confirmed a substantial (~3-fold) increase in elastin mRNA levels in cultures treated for 24 h with 20 μM iron and a significant reduction in tropoelastin mRNA in cultures maintained in the presence of 200 μM iron (Fig. 2B, right panel). Thus, results of both experiments demonstrated that different concentrations of iron may differently affect the steady-state levels of tropoelastin mRNA.

Because steady-state mRNA levels reflect the balance between transcription efficiency and message decay, we further human skin fibroblast cultures maintained in the presence of 60 μM DRB along with or without 20 and 200 μM of iron (as FAC) for 0, 6, 12, and 24 h. At indicated time points, total RNA was extracted and subjected to quantitative TaqMan RT-PCR analysis. Cultures treated with 200 μM iron (half-life of ~6 h) demonstrated~2.5-fold decrease in elastin message stability compared with untreated and 20 μM iron treated cultures (half-life of ~16 h). The results are expressed as the mean ± S.D. from two separate experiments conducted in quadruplicate cultures.
studied whether fluctuations in iron concentration may affect elastin mRNA stability. The stability of elastin message was determined in fibroblasts cultures simultaneously incubated with 60 μM DRB (a transcriptional inhibitor) in the presence or absence of either 20 or 200 μM of iron during a 24-h time-course period. The relative decay kinetics of elastin mRNA (quantified by real-time RT-PCR) was the same in control and 20 μM iron-treated cultures, with a half-life of ~16 h (Fig. 2C). In contrast, 200 μM iron-treated cultures demonstrated a rapid decrease in elastin mRNA level, which reached its half-life just...
after ~6-h incubation (Fig. 2C). These observations suggested that the treatment with high iron concentrations induce a decay in elastin mRNA levels.

Intracellular Iron Levels Influence Elastin Production—Because we have demonstrated that addition of low iron concentrations (up to 20 μM) to the culture media induced ~3-fold increase in elastin mRNA steady-state levels and subsequent increase in elastic fiber formation, we further tested whether this effect is specifically dependent on intracellular iron. To test this we utilized a highly specific membrane-permeable ferrie iron chelator, DFO, which have been shown to deplete intracellular pools of free iron (53–55). Results of immunocytochemistry (Fig. 3A), quantitative assay of newly deposited (metabolically labeled) insoluble elastin (Fig. 3B), and one-step RT-PCR analysis assessing elastin mRNA levels (Fig. 3C) demonstrated that chelating intracellular iron in cultured fibro-
blasts with 20 μM DFO significantly reduced elastin mRNA levels and consequent elastic fibers deposition, as compared with untreated control. Simultaneous treatment of cultured fibroblasts with equimolar amounts (20 μM) of ferric iron and DFO abolished the iron-induced increase in elastin mRNA levels and elastin deposition (Fig. 3). Cumulatively, these data indicate that chelatable intracellular iron facilitates normal expression of elastin gene and the consequent production of insoluble elastin.

The Effect of Iron on the Production of Intracellular ROS—It has been well established that iron has the capacity to generate ROS through the Fenton reaction and that ROS acting as second messengers may induce specific intracellular signaling pathways (56). We tested whether different iron concentrations that we utilized in our study may affect the production of ROS in normal human skin fibroblasts. Both fluorescence microscopy and flow cytometry measuring intracellular levels of ROS with a specific fluorescent probe showed that cells incubated with 2–40 μM iron produced the same amount of ROS as untreated controls. In contrast, the addition of higher concentrations of iron (100–400 μM) to the culture medium induced a dose-dependent increase in ROS production (Fig. 4).

Scavenging of Intracellular Hydroxyl Radical Reverts Inhibition of Elastin Production in Cells Treated with High Concentration of Iron—Because the above results indicate that the decrease in elastogenesis in cells treated with high concentrations of iron coincide with an increase in the production of intracellular ROS, we anticipated a pathophysiological link.
between these two effects. Results of the next series of experiments confirmed this hypothesis. We found that treatment of cultured fibroblasts with 200 μM iron and DMTU, the membrane-permeable scavenger of hydroxyl radicals (57), reversed the inhibitory effect of 200 μM iron treatment on elastin deposition (Fig. 5). In fact, 200 μM iron treatment in the presence of DMTU produced almost a 2-fold increase in elastin production as compared with cultures treated with 200 μM iron alone. We could not, however, detect a similar effect in cultures simultaneously treated with 200 μM iron, the membrane-impermeable antioxidants, catalase and superoxide dismutase (data not shown), and the membrane-permeable superoxide dismutase mimetic, Tempol (Fig. 5). We also found that pretreatment of cells with any of the four antioxidants prior to the addition of 20 μM iron did not change the stimulatory effect on elastin deposition (data not shown). These results further indicate that 20 μM iron treatment does not stimulate intracellular ROS production.

DISCUSSION

Impaired elastic fibers have been detected in patients with β-thalassaemia and in other inherited hemolytic disorders characterized with fluctuating iron levels (30, 32, 34, 58), however, no pathophysiological link between these two clinical features have ever been disclosed.

Mammalian cells maintain steady levels of metabolically active iron, also referred as the chelatable iron pool or labile iron pool (LIP), through the regulation of iron uptake and storage (59, 60), which is critical to maintaining normal cellular iron requirements (43). It has been shown that cells treated with lower than 25 μM iron (supplied as FAC) are able to maintain an equilibrium between LIP and iron bound to ferritin without a disturbance in cellular metabolism (45). Results of the present in vitro study demonstrate, for the first time, that treatment of normal human skin fibroblasts with such concentrations of iron can up-regulate tropoelastin synthesis and its final extracellular deposition into elastin fibers. Importantly, these low iron concentrations did not cause any increase in cellular proliferation rate (Fig. 1A). On the other hand, we have also shown that treatment of fibroblasts with elevated iron concentrations (100–200 μM FAC) slightly stimulated cellular proliferation but failed to further stimulate elastin production and in fact elicited an inhibitory effect.

The role of metal ions in transcription regulation has been documented for zinc (61, 62), copper (63), and calcium (64). It is becoming increasingly evident that fluctuations in iron levels...
can influence the expression of various genes through non-iron responsive element-mediated changes (36, 38, 65). Because we found that treatment of cultured fibroblasts with low iron concentrations (2–20 μM) caused 2- to 3-fold increase in the elastin mRNA level (Fig. 2B) and that the elimination of the LIP by treatment with a highly specific intracellular ferrox ferric chelator, DFO, led to a significant decrease in elastin mRNA levels and consequent elastin deposition (Fig. 3), we concluded that low intracellular concentrations of chelatable iron may facilitate normal elastogenesis. These data strongly indicate a new level of complexity to the poorly explored area of elastin gene regulation. However, the precise iron-dependent mechanism responsible for up-regulation of elastin gene transcription remains to be elucidated.

Using the analogy to the iron-dependent mechanism suggested for the activation of other genes such as protein kinase C-β (37), we speculate that certain iron-responsive transcriptional regulatory elements could be located within the elastin 5′-flanking region. However, to date only one true activating sequence has been identified within the elastin promoter, the nuclear factor-1 (NF-1) binding sequence, which, upon the interaction with one of the NF-1 family members, can directly activate elastin gene transcription (16). In separate studies a newly identified nuclear protein, pirin, has been shown to bind to NF-1 and was proposed as a functional cofactor for regulating gene transcription at the level of DNA complexes (66). Because pirin has recently been demonstrated to contain an iron-binding domain that is required for its function (67), we could speculate that the iron-induced increase in elastin message level would result from the pirin-dependent activation of NF-1 and consequent up-regulation in elastin gene transcription. However, more studies are needed to confirm this hypothesis.

The present study was designated to elucidate the cellular mechanism by which heightened iron concentrations might affect elastogenesis. Previously published data (45) indicated that treatment with elevated iron concentrations (100–200 μM FAC) causes an expansion in the LIP that cannot be buffered by the sequestration capacity of cellular ferritin. The high iron concentration (100–200 μM) utilized in the present study was comparable to concentrations used in other investigations of iron overload (41, 46). Our experimental conditions were also relevant to iron overload in β-thalassemia patients, where the levels of non-transferrin-bound iron rise substantially (68, 69). The non-transferrin-bound iron and its intracellular counterpart, the LIP, are able to catalyze the formation of the most toxic hydroxyl radicals through the Fenton and Haber-Weiss reactions (43, 70). The link between elastic fiber defects reported in chronic hemolytic syndromes and iron overload-induced oxidative stress have been previously suggested but not documented (30, 32, 34, 71, 72). Speculations about the involvement of oxidative stress in the development of elastic fiber pathology present in patients who suffer from inherited pseudoxanthoma elasticum, characterized with genetic deficiency of the MRP6 transporter (also needed for transport of reduced glutathione conjugates, free radical scavengers (73), have been raised by previous investigators (74) but not proven.

Results of our study provide the anticipated experimental evidence that the expansion of the intracellular LIP in cultured fibroblasts, treated with high concentrations of iron, result in a significant rise in intracellular levels of hydroxyl radicals, and the consequent decrease in elastic fiber formation. The fact that scavenging intracellular hydroxyl radicals with DMTU induced by 200 μM iron treatment leads to restoration of elastin deposition (~2-fold increase over untreated control, Fig. 5), confirmed our hypothesis that iron overload may impair elastogenesis.

It has been previously documented that ROS may alter the expression of certain genes by interfering with message stability (75–78). Our present data provide evidence that the iron-dependent generation of ROS indeed coincided with a decrease in the stability of elastin mRNA (Figs. 2C and 5). Although several mechanisms for regulating the stability of mRNAs have been described, only a few have been well characterized. In general, removal of 5′-cap structures or 3′-polyadenosine tails are considered to lead to rapid degradation of messages (79, 80). Sequence elements in the 3′-untranslated region have also been implicated in regulation of the stability of many mRNAs (81–84). Although the stability of elastin mRNA appears to be an important factor in regulating the expression of this protein, which has been reported to be affected by transforming growth factor-β (17, 85), phorbol esters (86), and vitamin D (87, 88), details of the mechanism of this regulation are still not understood.

Conserved GA-rich sequences present in the 3′-untranslated region of elastin have been shown to be an important element in the regulation of elastin mRNA stability (18). The presence of this sequence has been shown to be particularly susceptible to RNase attack when this site is not protected by yet unidentified binding protein factor(s) (18). We speculate that ROS might alter the binding of this putative protein to the GA-rich sequence in the 3′-untranslated region of elastin mRNA and consequently allow RNase to attack. Alternatively, elastin mRNA might be directly affected by oxidants or oxidant-dependent signaling molecules stimulating its degradation.

Despite the fact that our current study did not identify the one or more putative iron-dependent factors modulating elastin gene expression or stability of elastin message, the presented data clearly indicate that iron overload-induced oxidative stress interferes with elastogenesis. Importantly, we also demonstrate that the inhibitory effect of free radicals on elastogenesis can be minimized or eliminated by utilization of cell membrane-permeable antioxidants. We therefore suggest that further studies with these reagents would lead to development of novel therapeutic approaches for patients characterized with elevated iron levels and elastinopathy.

In conclusion, the present study, for the first time provides experimental evidence that intracellular chelatable iron can facilitate elastin gene expression resulting in abundant production of elastic fibers. Our data also indicate the existence of a parallel mechanism, in which an excess of intracellular chelatable iron induces the formation of free radicals that, through an unknown molecular manner, down-regulate elastin message stability and consequently decrease elastogenesis (Fig. 6). We suggest that an apparent balance between these two iron-dependent mechanisms may constitute a novel level of complexity regulating normal elastogenesis. We also believe that a disturbance of this balance, caused either by increased levels of free iron or chelation of intracellular iron, may result in impaired elastin production as observed in human hemolytic disorders.

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REFERENCES

1. Uitto, J., Hsu-Wong, S., Katchman, S. D., Bashir, M. M., and Rosenbloom, J. (1995) Ciba Found. Symp. 192, 237–256
2. Verheij, B., and Weiss, A. S. (1988) Eur. J. Biochem. 178, 1–18
3. Roark, M. H., Aune, D. D., Haendelschild, C. C., Godyna, S., Little, C. D., and Argraves, W. S. (1995) J. Histochem. Cytochem. 43, 401–411
4. Gilsen, M. A., Hatziinikolas, G., Kumaratilake, J. S., Sandberg, L. B., Nicholl, J. K., Sutherland, G. R., and Cleary, E. G. (1996) J. Biol. Chem. 271, 1096–1103
5. Kielty, C.; Badlock, C., Lee, D., Rock, M. J., Ashworth, J. L., and Shuttleworth, A. (2002) Philos. Trans. R. Soc. Lond. B Biol. Sci. 357, 207–217
6. Nakamura, T., Lozano, P. R., Ikeda, Y., Iwanga, Y., Hinek, A., Minamisawa, S., Cheng, C.-F., Kobuke, K., Dalton, N., Takada, Y., Tashiro, K., Ross, J., Honjo, T., and Chien, K. R. (2002) Nature 415, 171–175
