FUNCTIONAL DEFICIENCY IN THE 67-KD ELASTIN BINDING PROTEIN IS A CRUCIAL COMPONENT OF THE PATHOMECHANISM OF COSTELLO SYNDROME

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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Costello syndrome is characterized by mental retardation, loose skin, coarse face, skeletal deformations, as well as cardiomyopathy and predisposition to numerous malignancies. The genetic origin of Costello syndrome has not yet been defined. Using immunohistochemistry and metabolic labeling with [³H]-valine, we have established that cultured skin fibroblasts obtained from patients with Costello syndrome did not assemble elastic fibers despite an adequate synthesis of tropoelastin and normal deposition of the microfibrillar scaffold. We found that impaired production of elastic fibers by these fibroblasts is associated with a functional deficiency of the 67-kD elastin binding protein (EBP), normally required to chaperone tropoelastin through the secretory pathways and to its extracellular assembly. Metabolic pulse labeling of the 67-kD EBP with radioactive serine and further chase of this tracer indicated that both normal fibroblasts and fibroblasts from Costello syndrome patients initially synthesized comparable amounts of this protein, however, the fibroblasts from Costello syndrome patients quickly lost it into the conditioned media. Since the normal association between EBP and tropoelastin can be disrupted upon contact with galactosugar-bearing moieties, and the fibroblasts from Costello syndrome patients revealed an unusual accumulation of chondroitin sulfate-bearing proteoglycans (CD44 and biglycan), we postulate that a chondroitin sulfate may be responsible for shedding EBP from Costello cells and in turn for their impaired elastogenesis. This was further supported by the fact that exposure to chondroitinase ABC, an enzyme capable of chondroitin sulfate degradation, restored normal production of elastic fibers by fibroblasts from Costello syndrome patients. We also present evidence that loss of EBP from fibroblasts of Costello syndrome patients is associated with an unusually high rate of cellular proliferation.
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# TABLE OF CONTENTS

COSTELLO SYNDROME ................................................................. 1

THE DISCOVERY OF COSTELLO SYNDROME ..................................... 1
THE NATURAL HISTORY OF COSTELLO SYNDROME ............................... 2
HIGH INCIDENCE OF NEOPLASTIC GROWTH ASSOCIATED WITH COSTELLO SYNDROME ................................................... 5
THE GENETIC CAUSE OF COSTELLO SYNDROME HAS NOT BEEN IDENTIFIED AND THE METHOD OF TRANSMISSION IS STILL UNKNOWN .......................................................... 6
HISTOLOGIC EVALUATIONS SUGGEST IMPAIRED ELASTOGENESIS IN COSTELLO SYNDROME ................................................. 7

ELASTIC FIBERS ............................................................................. 8

COMPOSITION AND DISTRIBUTION .................................................. 8
MICROFIBRILS .............................................................................. 8
SYNTHESIS OF TROPOELASTIN AND ITS POST-TRANSLATIONAL MODIFICATIONS ............................................................ 9
ROLE OF THE ELASTIN BINDING PROTEIN IN ELASTOGENESIS ......... 10
EXCESS GALACTOSUGARS: DISRUPTION OF ELASTOGENESIS ............... 14
CROSS-LINKING ........................................................................... 18
DEGRADATION ............................................................................. 18
DISEASES OF ELASTOGENESIS ..................................................... 19
EBP AS A RECEPTOR ..................................................................... 22
RATIONALE AND AIMS .................................................................. 24

MATERIALS AND METHODS .......................................................... 25

FIBROBLAST CULTURES ............................................................... 25
IMMUNOSTAINING ........................................................................ 26
TROPOELASTIN AND INSOLUBLE ELASTIN ASSAYS ......................... 27
ISOLATION OF EBP ....................................................................... 28
ASSESSMENT OF GLYCOSAMINOGLYCAN METABOLISM ................. 29
ASSESSMENT OF FIBROBLAST PROLIFERATION ............................... 30

RESULTS ...................................................................................... 32

COSTELLO SYNDROME FIBROBLASTS PRODUCE EXTRACELLULAR MATRIX THAT LACKS ELASTIC FIBERS ................................. 32
COSTELLO SYNDROME FIBROBLASTS PRODUCE NORMAL AMOUNT OF TROPOELASTIN, BUT EXPERIENCE PROBLEMS WITH ITS SECRETION AND EXTRACELLULAR ASSEMBLY .................................................. 32
COSTELLO SYNDROME FIBROBLASTS PRODUCE NORMAL EBP, BUT IT IS RAPIDLY SHED FROM THEIR CELL SURFACE .......................................................... 43
COSTELLO SYNDROME FIBROBLASTS EXPRESS INTRACELLULAR AND PERICELLULAR ACCUMULATION OF CHONDROITIN SULFATE-BEARING MOIETIES .................................................. 48
ENZYMATIC REMOVAL OF CHONDROITIN SULFATE-BEARING MOIETIES FROM THE CELL SURFACE IMPROVES ELASTOGENESIS IN CULTURED COSTELLO SYNDROME FIBROBLASTS .................................................. 57
COSTELLO SYNDROME FIBROBLASTS PROLIFERATE FASTER THAN THEIR NORMAL COUNTERPARTS ................................. 64
EXPOSURE TO EXOGENOUS INSOLUBLE ELASTIN REVERSES THE ABNORMALLY HIGH PROLIFERATION OF COSTELLO SYNDROME FIBROBLASTS .............................................. 64

DISCUSSION .................................................................................. 69

REFERENCES .................................................................................. 75
# TABLE OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Costello syndrome patient</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Schematic of elastic fiber assembly</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Galactosugar interaction with Elastin Binding Protein</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>Diseases of elastogenesis</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Morphometric analysis of extracellular matrix components</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>Lysyl oxidase and Fibrillin type 1 immunocytochemistry</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>Collagen type 1 and fibronecin immunocytochemistry</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>Immunocytochemical and biochemical assessment of elastin</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>Biochemical assessment of total and secreted tropoelastin</td>
<td>42</td>
</tr>
<tr>
<td>10</td>
<td>Biochemical assessment of intracellular tropoelastin</td>
<td>45</td>
</tr>
<tr>
<td>11</td>
<td>Elastin binding protein immunocytochemistry</td>
<td>47</td>
</tr>
<tr>
<td>12</td>
<td>Elastin binding protein pulse and chase</td>
<td>50</td>
</tr>
<tr>
<td>13</td>
<td>Monoclonal chondroitin sulfate immunocytochemistry</td>
<td>52</td>
</tr>
<tr>
<td>14</td>
<td>CD-44 immunocytochemistry</td>
<td>54</td>
</tr>
<tr>
<td>15</td>
<td>Biglycan immunocytochemistry</td>
<td>56</td>
</tr>
<tr>
<td>16</td>
<td>Peanut agglutinin histochemistry</td>
<td>59</td>
</tr>
<tr>
<td>17</td>
<td>Sulfate incorporation</td>
<td>61</td>
</tr>
<tr>
<td>18</td>
<td>Elastic fiber restoration with chondroitinase ABC</td>
<td>63</td>
</tr>
<tr>
<td>19</td>
<td>Proliferation phase contrast micrographs</td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>Cell proliferation assays</td>
<td>68</td>
</tr>
</tbody>
</table>
Costello Syndrome

The discovery of Costello syndrome

In 1977, Costello described 2 unrelated children with mental subnormality, poor postnatal growth and nasal papillomata as a new clinical entity (Costello 1977). Additional case reports of similar patients did not appear until 1991 (Der Kaloustian et al 1991; Martin and Jones 1991; Berberich et al 1991) when the list of clinical symptoms was expanded to include short stature, redundant skin of the neck, palms, soles and fingers, hyperkeratotic palms and soles, curly hair, aged appearance, epicanthal folds, large depressed nasal bridge, and large earlobes. Despite this, numerous diagnoses including cutis laxa (Patton and Baraitser 1993; Davies and Hughes 1994a, 1994b; Vila Torres et al 1994), Donohue and Leprechaun syndromes, Berardinelli-type lipodystrophy (Zampino et al 1993), Noonan or cardio-facio-cutaneous syndromes (Borochowitz et al 1992; Teebi and Shaabani 1993; Fukushima 1996; Wieczorek et al 1997) and ectodermal dysplasias (van Eeghen et al 1999) have been initially considered in patients demonstrating the clinical features of the syndrome described by Costello. There are now more than 40 papers (see review, van Eeghen et al 1999) describing patients with this rare and distinct clinical entity, officially named the Costello syndrome (Costello 1996, Fukao et al 1996, van Eeghen et al 1999). However, there are no reports that address the molecular or biochemical basis of the disease.

The natural history of Costello syndrome

The natural history of Costello syndrome is characterized by two distinct phases. The first phase is often marked by polyhydramnios and increased birth weight and
followed by the second phase which includes: failure to thrive, severe short stature, mental retardation, and a distinctive appearance (fig. 1) with craniofacial and dermatologic findings resembling those observed in lysosomal storage disorders (Berberich et al 1991). Affected individuals may have a silent or clinically significant hypertrophic cardiomyopathy, cardiac valve malformations and dysrhythmia (Fukao et al 1996; Siwik et al 1998; Tomita et al 1998). Other manifestations of Costello syndrome include soft skin with excess wrinkling over the dorsum of the hands and deep creases on the palms and soles, hyperextensibility of digits, generalized hyperpigmentation, pigmented nevi, vascular birthmarks, acanthosis nigricans, occasionally sialuria (Di Rocco et al 1993), papillomata and other tumors that develop at later ages (Say et al 1993; Costello 1996; Philip and Sigaudy 1998; Suri and Garrett 1998; Kerr et al 1998; Feingold 1999; Francechini et al 1999; Gripp et al 2000).

Congenital heart defects are common in Costello syndrome. It has been reported that 60% of patients with Costello syndrome have at least one cardiac abnormality. Most patients have a systolic heart murmur and heart dysrhythmias were also common (Siwik 1998). Furthermore, 30% have structural heart disease, 65% have been reported to have hypertrophic cardiomyopathy with valve dysfunction, and 18% have tacharyrhythmia (Johnson et al 1998; Siwik et al 1998). Specifically, five patients were reported to have pulmonary stenosis (Berberich et al 1991; Borochowitz et al 1992; Pratesi et al 1998), five had ventricular septal defects (Teebi and Shaabani 1993; Di Rocco et al 1993; Kondo et al 1993; Siwik et al 1998), three had a thickening of the intraventricular septum and tips of the mitral valves (Izumikawa et al 1993; Davies and Hughes 1994b; Siwik et al 1998), two an atrial septal defect (Say et al 1993; Pratesi et al 1998), one a persistent
Figure 1

Photographs depicting a patient with Costello syndrome at 6-months of age, whose fibroblasts were used in the presented studies. Note: (A) sparse and curly hair, "coarse" face, depressed nasal bridge, bulbous and upturned nose, low-set ears, full cheeks, large tongue, pouting lower lip, strabismus, and deep palmar (B) and plantar (C) creases.
ductus arteriosus (Borochowitz et al 1992), one subpulmonic stenosis (Siwik et al 1998), and one mitral valve prolapse (Martin and Jones 1991).

**High incidence of neoplastic growth associated with Costello syndrome**

Initial descriptions of patients with Costello syndrome listed the presence of papillomata or wart-like lesions around the mouth and nose as one of the common features. Recent comprehensive reviews of Costello syndrome patients indicated that they display numerous neoplastic growths in addition to papillomata (van Eeghen et al 1999; Ker et al 1998). This hypothesis has been reinforced by recent publications concerning neoplasms of different origins in Costello syndrome patients: two reports of bladder carcinoma (Franceschini et al 1999; Gripp et al 2000), a child with an alveolar rhabdomyosarcoma of the right foot at 6 months of age (Feingold 1999), two children with retroperitoneal embryonal rhabdomyosarcoma that developed at 1 month of age in both patients (Kerr et al 1998), and a Costello patient who died at 33 years of age with a ventricular schwannoma (Suri and Garrett 1998). Kerr and associates recently suggested (1998) that an increased incidence of neoplastic growth could be associated with the clinical picture in Costello syndrome. If there is an increased incidence of neoplasms this suggests that mechanisms controlling cell growth and differentiation may be affected in Costello syndrome patients.
The genetic cause of Costello syndrome has not been identified and the method of transmission is still unknown.

Existence of two families with affected siblings (Berberich et al 1991; Zampino et al 1993) and three consanguineous matings (Borochowitz et al 1992; Franceschini et al 1999) led to the hypothesis that autosomal recessive inheritance was the likely mode of transmission of Costello syndrome. The families that were reported by Borochowitz and associates (1992), however, were from an area where consanguinity is common. A linkage analysis performed by Lurie (1994) reported that only 2 of a possible 46 siblings of Costello patients were affected (Lurie 1994; Czeizel and Timar 1995; Fukao et al 1996; Pratesi et al 1998). This makes the hypothesis of autosomal recessive inheritance unlikely and sporadic autosomal dominant mutations as the likely cause. The cases of Costello syndrome occurring in siblings could be explained by germline mosaicism, however, it has also been suggested that Costello syndrome is a heterogeneous syndrome with various modes of inheritance. Currently, the inheritance of Costello syndrome seems likely to be caused by de novo mutations in a single gene or potentially a microdeletion syndrome (contiguous gene syndrome), causing a loss- or gain-of-function mutation potentially causing the disruption of a regulatory system (van Eeghen et al 1999; Czeizel and Timar 1995). There is one single report of a translocation event in a Hungarian girl with Costello syndrome: 46,XX t(1;22)(q25;q11) (Czeizel and Timar 1995). Suri and Garrett (1998) also reported a patient with neurofibromatosis type 2 (NF2) which is localized to chromosome 22q, however, they found no evidence of a translocation in the NF2 region on chromosome 22, indicating that Costello syndrome may only be associated to chromosome 22.
Histologic evaluations suggest impaired elastogenesis in Costello syndrome

Qualitative and quantitative histological analyses of the skin of patients with Costello syndrome demonstrated impaired elastin deposition (Vila Torres et al 1994). In fact, an abnormal size, shape, and arrangement of elastic fibers in patients with Costello syndrome resembled those described in cutis laxa. Histopathologic studies of autopsy cases revealed that impaired deposition of elastin also occurs in the tongue, pharynx, larynx and upper esophagus of patients with Costello syndrome, and that elastic fibers of the bronchi, alveoli, aorta and coronary arteries of these children are thinner than in normal patient tissue specimens (Mori et al 1996). Despite the fact that some patients with Costello syndrome resemble those with William’s syndrome, genetic analysis of their cells did not reveal any deletion of the elastin gene and demonstrated normal expression of elastin mRNA (Mori et al 1996). This suggested that the observed disruption of elastic fibers in tissues of patients with Costello syndrome may be due to either post-transcriptional or post-translational modifications of tropoelastin message and/or to problems arising from its inadequate secretion and extracellular deposition of this protein.

Since fully functional elastic fibers are an essential component of normal connective tissues we hypothesize that their disruption observed in Costello syndrome may be directly related to the pathomechanism of this disease characterized by multiple connective tissue disorders.
Elastic fibers

Composition and Distribution

Elastic fibers are present in the extracellular matrix of the connective tissues. They provide the resilient framework of blood vessels, lungs, skin and other organs. Elastic fibers are composed of: the amorphous elastin, and parallelly orientated 10-12 nm microfibrils. Microfibrils are made up of two distinct proteins called fibrillins, two different microfibril associated glycoproteins (MAGPs), and other accessory components such as, lysyl oxidase, elastin binding protein, osteopontin, emilin, fibulin-1 and proteoglycans (Vrhovski and Weiss, 1998).

Formation of elastic fibers - elastogenesis - begins with the production of elastin’s soluble precursor, called tropoelastin. Molecules of monomeric 70-kD tropoelastin, synthesized by such cells as fibroblasts, chondrocytes, and smooth muscle cells, have to be shuttled through the secretory pathways and brought into close association with the microfibril scaffold in the extracellular space. The proper and orderly assembly of tropoelastin molecules on the microfibrillar scaffold is absolutely prerequisite for their subsequent cross-linking by lysyl oxidase (fig. 2) (Mecham and Hauser 1991; Zhang et al 1994a). Elastogenesis is a multistep process that is modulated by several other factors whose roles are just emerging and not fully understood.

Microfibrils

Microfibrils are assembled in the extracellular space prior to the secretion of tropoelastin. The microfibrils are composed of heterogeneous glycoproteins such as fibrillin type 1, fibrillin type 2 and microfibril-associated glycoprotein (MAGP) (Mecham
and Hauser 1991; Parks et al 1993; Rosenbloom et al 1993; Christiano and Uitto 1994; Vrhovskii and Weiss 1998; Debell and Tamburro 1999). Fibrillin aligns in a head to tail fashion as parallel bundles of 6 to 8 molecules to form the major structural component of the microfibrils (Ramirez and Pereira 1999) which also include MAGP’s and proteoglycans (Vrhovskii and Weiss 1998). It has been shown that both fibrillins have N-terminal domains that are able to associate with tropoelastin. Both, the C-terminal domain of tropoelastin and the domain encoded by exon 30 have been identified as microfibril-binding motifs (Brown-Augsberger et al 1994; Brown-Augsberger et al 1996).

**Synthesis of Tropoelastin and its Post-Translational Modifications**

Smooth muscle cells, endothelial cells, microvascular cells, chondrocytes and fibroblasts have all been found to synthesize tropoelastin (Uitto et al. 1991). The message for this soluble precursor of elastin is encoded by the 34 exon-long elastin gene and translated to 70-kD monomers (Indik et al 1987). Tropoelastin is composed of two alternating types of domains: the hydrophobic and the hydrophilic. The hydrophobic domains are rich in Glycine, Valine, Proline and Alanine that occur many times in repeats such as GVGVP, GGVP and GVGVAP (Pasquali-Ronchetti and Baccarani-Contri 1997). Usually alternating the hydrophobic domains are the hydrophilic AKAAAAKAAAAKA domains essential for future cross-linking. The N-terminus of tropoelastin contains a 26 amino acid signal peptide and the highly conserved C-terminus containing two cysteine residues and a positively charged RKRK sequence.
Translation of the tropoelastin mRNA takes place on the surface of the rough endoplasmic reticulum (RER) and the polypeptide chains are released into the lumen of the RER with the release of the signal peptide (Saunders and Grant 1984). Tropoelastin undergoes very little post-translational modification. There is no evidence that the mature product undergoes any glycosylation, however, hydroxylation may take place. Hydroxylation occurs to a variable degree (0-20%) of the total proline residues but it appears that hydroxylation is not necessary for elastic fiber synthesis (Uitto et al. 1991).

**Role of the Elastin Binding Protein in Elastogenesis**

The mechanisms governing tropoelastin secretion and assembly are not yet fully elucidated. The current data indicate that highly hydrophobic and non-glycosylated tropoelastin has to bind to several intracellular proteins that chaperone it through the intracellular compartments. Davis and colleagues (1998) documented that in endoplasmic reticulum tropoelastin binds to BIP, an HSP70 molecular chaperone and the peptidyl-prolyl cis-trans isomerase, FKPB65. Immunohistochemical and biochemical studies indicated that tropoelastin, present in endosomal and Golgi compartments, is escorted by the 67-kD elastin binding protein (EBP) (Hinek et al., 1988; Hinek and Rabinovitch 1994; Hinek et al. 1995), which protects tropoelastin from premature intracellular self-aggregation and proteolytic degradation (Hinek and Rabinovitch 1994). We have established that after delivering tropoelastin extracellularly to the growing elastic fibers the EBP molecules dissociate from tropoelastin and returns to the endocytic compartments (but not to the lysosomes). Once in the endocytic compartment EBP may bind again to newly synthesized tropoelastin in the trans-Golgi network, and recycle back
to the cell surface (Hinek et al 1995). Of particular importance was the observation that 67-kD EBP also has a separate galactolectin domain and it has been suggested that binding of galactosugars to this domain induces conformational changes in EBP, resulting in its dissociation from tropoelastin (Hinek et al 1988; Mecham et al 1989; Hinek et al 1991). This led to the paradigm, that the release of newly secreted tropoelastin molecules from their EBP transporters, occurs on the cell surface, upon the interaction between EBP and galactosugar moieties, presumably those protruding from carbohydrate chains of glycoproteins forming the microfibrillar scaffold of the growing elastic fibers (fig. 2).

Partial sequencing of EBP isolated from sheep smooth muscle cells led to the discovery that EBP is identical to the 67-kD enzymatically inactive variant of human β-galactosidase (Hinek et al. 1993). The message encoding this 67-kD EBP is an alternatively spliced variant of β-galactosidase (S-gal) (Privitera et al 1998) arising through alternative splicing of β-galactosidase mRNA of two non-contiguous protein encoding regions (Hinek 1996). The splicing of the first region induces a frame shift that is restored by the deletion of the second. The resulting encoded protein still contains a galactosugar binding domain, but apparently loses the enzymatic activity and lysosomal targeting, and gains a unique 32 amino acid long frame-shift generated sequence that contains the VVGPSAQLDEASPL elastin binding domain (Hinek 1994; Hinek 1996).
Figure 2
Schematic illustrating the process of elastic fiber assembly. Tropoelastin is shuttled through the secretory pathways from the golgi to the extracellular space by the elastin binding protein (EBP). EBP then interacts with microfibrils releasing tropoelastin into the growing elastic fiber. EBP is then able to recycle through the endosomal compartment to shuttle a new tropoelastin molecule to the extracellular space.
Elastic Fiber Assembly

Glycoprotein Scaffold:
Fibrillin, MAGP

Crosslink

Plasma Membrane

Secretory vesicles

Elastin Binding Protein
Excess Galactosugars: Disruption of Elastogenesis

The current model of extracellular assembly of elastic fibers proposes that tropoelastin must be released from EBP via interaction of galactosugars, likely protruding from the microfibrillar glycoproteins at the correct spatial and temporal location (Hinek et al 1991; Hinek 1994, 1996). It has been established, however, that addition of exogenous free galactosugars (galactose, lactose) or pericellular accumulation of endogenous galactosugar-bearing moieties (dermatan sulfate, chondroitin sulfate, keratan sulfate) can disrupt the process of orderly elastogenesis. These moieties may simply bind to the tropoelastin-bearing EBP and lead to a premature release of tropoelastin far away from the assembly sites localized on the microfibrillar scaffold (fig. 3) (Hinek et al 1988, 1991, 1992, 1993; Hinek 1996).

The galactosugar-dependent shedding of the EBP and consequent impaired elastogenesis has previously been linked to the developmental mechanism responsible for physiological closure of fetal ductus arteriosus (DA) and to pathological intimal thickenings observed in arterial diseases. The DA is a small shunt vessel between fetal aorta and pulmonary artery that closes immediately after birth. Closure occurs after formation of intimal cushions in this vessel. During this period, DA cells produce two-fold more chondroitin sulfate than aortic smooth muscle cells (SMCs) (Boudreau and Rabinovitch 1991) and at the same time they do not assemble elastic fibers. Since production of elastic fibers is disrupted, medial SMC are able to migrate toward the intima and proliferate. Extensive migration and proliferation of medial SMC that have detached from surrounding elastic lamellae and are not capable of normal elastogenesis
Figure 3

Interaction of galactosugars (lactose, galactose, chondroitin sulfate, N-acetylglucosamine) with the elastin binding protein’s (EBP) lectin domain causes a conformational change in EBP that results in its dissociation from tropoelastin and the cell surface.
are an important constituent part of the pathomechanism in occlusive arterial disease (Boudreau and Rabinovitch 1991).

Furthermore, a similar mechanism of elastic fiber disruption has been shown in Hurler syndrome, a lysosomal storage disease that arises out of a deficiency in the enzyme \( \alpha \)-L-iduronidase and results in the accumulation of dermatan and heparan sulfate proteoglycans. The inability of the lysosomes to properly degrade the dermatan and heparan sulfate glycosaminoglycans causes them to accumulate not only intracellularly, but also in the pericellular space. The increased levels of dermatan sulfate glycosaminoglycans interacting with EBP on the cell surface have been proposed as the factors causing shedding of EBP and a consequent disruption of elastogenesis in this storage disease (Hinek and Wilson, 2000). In addition to dermatan sulfate, we have also shown that \( N \)-acetylgalactosamine residues present in chondroitin sulfate glycosaminoglycans can also interact with the galactolectin domain on EBP, and disrupt elastogenesis (Hinek 1994, 1996; Hinek et al 1993, 2000; Hinek and Wilson; 2000). Thus, it is reasonable to speculate that connective tissue disorders observed in certain mucopolysacharidoses (genetic errors causing deficiency of lysosomal enzymes responsible for glycosaminoglycans degradation) may also be linked to impaired elastic fiber formation caused by loss of EBP induced by an abnormal storage of chondroitin and dermatan sulfate glycosaminoglycans.
Cross-Linking

The final stages in elastogenesis require the cross-linking activity of the copper-dependent lysyl oxidase (Kagan and Sullivan 1982). After tropoelastin is secreted into the extracellular space and properly associates with the microfibrils, it is rapidly rendered insoluble by cross-link formation without any further modifications or proteolytic processing (Bressan and Prockop 1977). The initial reaction in cross-link formation is an oxidative deamination by the enzyme lysyl oxidase to form allysine. The subsequent reactions occur spontaneously and eventually form the tetrafunctional desmosine and isodesmosine unique to elastin (Reiser et al 1992; Mariani et al 1992). Lysyl oxidase can be incorporated into growing elastic fibers, accounting for most lysine residues being cross-linked to form desmosines or isodesmosines. The high level of lysine cross-links in the elastic fiber account for its extreme insolubility and resistance to degradation.

Degradation

Insoluble elastin has a very slow turnover in normal tissue and can take several years to be degraded in humans barring any injury or trauma (Hall and El-Ridi 1976). This is largely due to the fact that insoluble elastin is highly resistant to proteolytic degradation. Degradation of elastin is primarily achieved by elastases, which can also degrade a number of other extracellular matrix proteins. The degradation of an excess elastin is important in such physiological processes as tissue remodelling during growth and uterus involution after pregnancy (Werb et al 1982). Inappropriate and uncontrolled elastolysis can be destructive and facilitate the development of cardiovascular diseases,
emphysema and can also accelerate normal ageing of the skin and ligaments (Braverman and Fonferko 1982).

**Diseases of Elastogenesis**

The biosynthesis of tropoelastin is the product of a single gene at 7q11.1-21.1 in the human. Due to the complexity and number of factors involved in the formation of the elastic fiber, defects in the production of tropoelastin only account for a small proportion of the diseases of elastogenesis. Supravalvular aortic stenosis (SVAS), William’s syndrome and congenital cutis laxa have been directly linked with alterations in the elastin gene (Lowery et al 1995; Zhang et al 1999). However, there are many diseases of elastogenesis associated with defects in the components and enzymes responsible for assembly of the elastic fiber. For example, mutations in the fibrillin-1 gene are responsible for Marfan’s syndrome, defects in the copper transport protein Mc-1 result in Menkes syndrome, and defects in the elastin cross-linking enzyme lysyl oxidase result in Ehlers-Danlos syndrome (fig. 4) (Vrhovski and Weiss 1998). Acquired diseases involving elastin also include emphysema, which results from increased elastin degradation in the lung and atherosclerosis where a loss of elasticity is accompanied by calcium and lipid deposition.

Recent data from our laboratory clearly indicate that several other diseases caused by primary genetic errors involving enzymes normally responsible for proteoglycan degradation are also characterized by impaired elastogenesis. We found that cells from Morquio B disease patients and certain patients with GM1-gangliosidosis bearing
Figure 4

Schematic representation of diseases that can disrupt the multi-step process of elastogenesis. Each of the diseases shown is characterized by a lack of insoluble elastin in the extracellular matrix due to a primary or secondary deficiency in one of the necessary proteins involved in the formation of elastic fibers.
Diseases of Elastogenesis

**Ehlers-Danlos Syndrome:**
Defective lysyl oxidase is unable to cross-link elastin

**Hurler Syndrome:**
Proteoglycan accumulation causes premature shedding of EBP

**Sialidosis and Galactosialidosis:**
Mutations cause defective neuraminidase and protective protein

**GM₁ Gangliosidoses**
(nonsense mutation):
β-galactosidase gene mutation, EBP not produced

**William’s Syndrome:**
Hemizygosity at the elastin locus

**Marfan’s Syndrome:**
Mutations in fibrillin gene cause defective scaffold
nonsense mutations of β-Gal gene, or mutations located in a coding region common for lysosomal β-galactosidase and S-Gal, do not produce EBP. Thus, due to a primary deficiency in this tropoelastin chaperone those cells are not capable of normal elastic fiber assembly (Hinek et al. in press). We have also established that secondary deficiency in S-Gal/EBP, due to its premature shedding caused by interaction with galactosugar-bearing moieties accumulating in pericellular space (Hinek and Wilson 2000), led to impaired elastogenesis. It is important to mention that Costello syndrome shares several cardinal clinical features (skeletal malformations, coarse face, multi-organ lesions of connective tissue with impaired elastic fibers) with the above-mentioned diseases already characterized with the primary or secondary S-Gal deficiency (Berberich et al. 1991).

**EBP as a Receptor**

In addition to EBP's chaperoning role, there have been reports that EBP works as a receptor that can modulate cell behaviour based on changes in the extracellular environment. EBP is expressed by leukocytes that show chemotactic activity towards degradation products of elastin (Hinek 1996). Cell cycle stimulation in astrocytoma cell lines by elastin degradation products show upregulation of certain cyclins indicating that EBP can modulate the cell cycle (Jung et al. 1998). Fibroblasts from patients with Hurler syndrome have also been shown to have an accelerated rate of proliferation. Addition of exogenous insoluble elastin has been shown to slow down rates of proliferation (Hinek and Wilson 2000). These results seem to indicate that EBP has the ability to modulate mitogenic signals dependent on the conditions of the extracellular environment.
In addition, it has been shown that EBP without tropoelastin on the cell surface has the ability to block the binding of interleukin-1 beta (IL-1β) with its receptor. The removal of EBP by the addition of exogenous CS or free galactosugars, such as lactose, increases the amount of IL-1β binding to its receptor and causes a subsequent upregulation of fibronectin synthesis (Hinek et al 1996).
Rationale and Aims

Reports of disrupted elastic fibrils in Costello syndrome, despite apparently normal tropoelastin mRNA (Vila Torres et al 1994; Mori et al 1996), indicate that some post-transcriptional stage of elastogenesis may be affected. Since disruption of elastic fiber production may arise either from low production of tropoelastin and microfibrillar proteins (e.g., William's and Marfan's syndromes respectively), or from their inadequate secretion and extracellular assembly (Hurler syndrome), the present study was aimed at assessing the major steps of elastogenesis in fibroblasts derived from six children with Costello syndrome and from three age-matched normal children.

This was in hope that eventual elucidation of pathomechanism responsible for impaired elastogenesis in Costello syndrome may provide insight into the origin of this rare disease. Initial studies also indicated that Costello fibroblasts produced normal amounts of tropoelastin and microfibrillar components, but showed low expression of the 67-kD EBP. Thus, it was hypothesized that a primary or secondary deficiency in EBP is responsible for impaired production of elastic fibers in Costello syndrome.
Materials and Methods

Fibroblast Cultures

Six children ranging in age from 1 month to 16 years were diagnosed with Costello syndrome at the Hospital for Sick Children (Toronto, Canada) (7669, 9951, 10595, 12195, 12196, 12368) based on the presence of a cardinal clinical features previously ascribed to this phenotype (Costello 1996, Johnson et al 1998, van Eeghen et al 1999). These features include characteristic coarse facies and thick lips, mental retardation, postnatal growth retardation, sparse and curly hair, deep palmar and plantar creases, loose skin of the hands and feet, hypertrophic cardiomyopathy and arrhythmia, papillomata and other tumours. Biopsies of forearm skin from three Costello syndrome patients (7669, 9951, 10595) as well as biopsies from three normal children (4212, 3858, 4184) of matching ages were used as the primary source of fibroblasts tested in immunohistochernical, biochemical and cell proliferation studies. Moreover, fibroblasts obtained from three other Costello syndrome patients (12195, 12196, 12368) were analyzed by immunohistochemistry alone. Fibroblasts were originally isolated by collagenase digestion of the skin fragments and then passaged 2-6 times by trypsinization and maintained in alpha-minimum essential medium supplemented with 20mM Hepes, 1% antibiotics/antimycotics, 10% fetal bovine serum (FBS). Alpha-Minimum Essential Medium, fetal calf serum, and other cell culture products were obtained from GIBCO Life Technologies (Burlington, ON).
Immunostaining

Subconfluent, 48-hour cultures of normal and Costello syndrome fibroblasts, fixed in cold 100% methanol at -20°C for 30 minutes, were incubated with 10 μg/ml of polyclonal antibody raised to the elastin/laminin binding domain of the alternatively spliced variant of β-galactosidase (anti-S-GAL) (Hinek et al 1993), and the BCZ monoclonal antibody which recognizes a different epitope on the EBP (Mecham et al 1988). Parallel cultures fixed for 10 minutes in 4% paraformaldehyde with or without detergent (0.2% Triton X-100) that permeabilizes cell membranes were treated with 10 μg/ml of monoclonal antibody to chondroitin sulfate (Avnur and Geiger 1984) (obtained from Sigma, St. Louis, MI) or with 10μg/ml of antibodies recognizing chondroitin sulfate-bearing proteoglycans, CD-44 and biglycan. Hermes-1 CD-44 antibody was obtained from Endogen (Cambridge, MA) and anti-biglycan (LF 112) was a generous gift of Dr. Larry Fisher, National Institute of Health (Bethesda, MD).

Additionally, the histochemical detection of galactosugar-bearing glycoconjugates was also performed by staining non-permeabilized and permeabilized fibroblasts with 0.1 mg/ml of fluorescein-conjugated peanut agglutinin (FITC-PNA), which selectively binds to Galβ1-3GalNAc domains and was obtained from EY Laboratories (San Mateo, CA) (Zhou et al 1999).

Ten-day-old cultures, containing abundant extracellular matrix, fixed in cold 100% methanol, were incubated with either 20 μg/ml of polyclonal antibody to tropoelastin, purchased from Elastin Products Co., Inc. (Owensville, MI) (Prosser et al 1991), with 2 μg/ml of monoclonal antibody to fibronectin purchased from ICN (Costa Mesa, CA).
(Vartio et al 1987) and with 10 μg/ml of polyclonal antibody to collagen type I (LF 39, Dr. Larry Fisher, NIH). The parallel cultures scheduled for immunohistochemical assessment of microfibrillar components were fixed in 0.5% paraformaldehyde for 15 minutes, blocked in PBS containing 0.1 M ammonium chloride, then treated with specific 20 μg/ml of polyclonal antibody to fibrillin, or additionally pretreated for 10 minutes with PBS containing 50 mM DTT, alkylated with 100 mM iodoacetamide for 15 minutes, washed in PBS and then immunostained with specific polyclonal antibody to MAGP at the same concentration. Antibodies to fibrillin and MAGP were obtained from Elastin Products Co., Inc. (St. Louis, MO). All cultures were incubated with appropriate fluorescein-conjugated secondary antibodies (GAR-FITC or GAM-FITC) for an additional hour. Secondary antibodies were obtained from Sigma (St. Louis, MO). Nuclei were counterstained with propidium iodide.

Morphometric analysis of 10-day-old cultures immunostained with antibodies recognizing extracellular matrix components was performed using an Olympus AH-3 microscope attached to a CCD camera (Optronics) and a computerized video analysis system (Image-Pro Plus software 3.0 for Macintosh, Media Cybernetics, Silver Spring, MD).

Tropoelastin and Insoluble Elastin Assays

Fibroblasts from Costello syndrome and normal patients were grown to confluency in 100 mm dishes in quadruplicates. [3H]-valine (20 μCi, from Amersham, Oakville, ON) was added to each dish along with fresh media. Cultures were then incubated for 72 hours, then the soluble and insoluble elastin was assessed separately in each culture as
previously described (Hinek and Rabinovitch 1994). Briefly, conditioned media was collected and immunoprecipitated with a polyclonal antibody to tropoelastin, then soluble proteins present in the intracellular compartments were extracted with 0.1 M acetic acid, intracellular tropoelastin was immunoprecipitated from extracts and quantitatively assessed by scintillation counting. The remaining cultures containing cell remnants and deposited insoluble extracellular matrix were scraped and boiled in 0.5 ml of 0.1N NaOH for 45 minutes to solubilize all matrix components except elastin. The resulting pellets containing the insoluble elastin were then solubilized by boiling in 200 μL of 5.7 N HCl for 1 hour and the aliquots were mixed with scintillation fluid and counted.

The integrity of tropoelastin produced by fibroblasts from normal and Costello syndrome patients was also assessed after confluent cultures (initially plated 5x10^6 cells/dish) were pulsed with 15 μCi/ml [14C]-valine (Amersham, Oakville, ON) in valine-free medium for 2 hours. The intracellular tropoelastin was then immunoprecipitated with specific anti-tropoelastin antibody (Prosser et al 1991) from the cell layer extracts produced with 1% Nonidet P-40 in 50 mM TBS pH 8 containing proteinase inhibitors (2 mM benzamidine, 2 mM EACA, 2 mM PMSF, 1 mM EDTA, 2 mM leupeptin and 1 mg/ml Trasylol), and analyzed by SDS-PAGE followed by autoradiography and by Western immunoblotting.

**Isolation of EBP**

To compare patterns of the EBP expression by fibroblasts from normal and Costello syndrome children, we carried out pulse-chase experiments. Fibroblasts were initially plated 1x10^6 cells/dish to form a subconfluent culture, incubated in triplicate in serum-
free Medium 199 for 6 hours, and then pulsed with 15 μCi/ml [14C]-serine in serine-free medium for 1 hour. The cultures were then rinsed well and chased in fresh Medium 199 for 5, 15, 30 and 45 minutes. At the end of each chase period, the cell layers and the media were processed separately. To isolate the EBP the standard elastin affinity chromatography technique was used as described previously (Hinek et al 1993, Hinek and Wilson 2000). The elastin bound proteins released from elastin slurries were resolved by 7.5-12% polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. The identity of EBP was additionally confirmed by immunoblotting with affinity purified anti S-Gal antibody recognizing 67-kD EBP, followed by GAR-HRP conjugated secondary antibody and amplification with the ECL chemiluminescence detection system. The media from each culture was also mixed with a protease inhibitor cocktail and then subjected to immunoprecipitation with 2 μg/ml of anti S-Gal antibody (Privitera et al 1998).

**Assessment of Glycosaminoglycan Metabolism**

Fibroblasts from normal and Costello syndrome children were suspended in alpha MEM containing 10% FBS and initially plated in 10 cm dishes in quadruplicate at an initial density of 2.5 x 10^5 cells per dish. The cells were incubated for 5 days and then the media was aspirated and replaced with sulfate depleted MEM supplemented with ^35[SO4] at a concentration of 20μCi per mL. The dishes were incubated for an additional 7 days before measurement of radioactive-sulfate incorporation began. Assessment of incorporation was performed according to Cantz and associates (1972). First, dishes were aspirated and rinsed with 0.9% NaCl. The cells were then detached with trypsin
and transferred to 15 mL conical tubes. The supernatants were centrifuged at room temperature for 4 minutes at 1000 g. The tubes were aspirated and rinsed with 0.9% NaCl, and then spun again for 4 minutes at 1000 g. After removal of the saline supernatant the pellets were washed with 80% ethanol and the cell pellets were broken up with a sealed Pasteur pipette. The tubes were then placed in a gently boiling water bath and removed and allowed to cool after the ethanol began to boil. After cooling, the tubes were boiled again and centrifuged at 1000g for 4 minutes. The ethanol was aspirated and fresh ethanol was added and again the cell pellets were broken up by a sealed Pasteur pipette. The boiling extraction process was repeated as before. After cooling, centrifugation and removal of the supernatant fluid, 1mL of 10% NaOH is added and the tubes are once again placed in the boiling water bath. After the precipitate has dissolved and cooled an aliquot is mixed with scintillation fluid and counted for radioactivity. Another aliquot (0.1mL) was neutralized with 1.5 volumes of 2 N acetic acid and used for protein determination.

Assessment of Fibroblast Proliferation.

Fibroblasts from normal and Costello syndrome children were suspended in alpha MEM containing 10% FBS and initially plated in 6 well-dishes at a density of 50 000 cells per well. The medium was changed 24 hours later, and parallel cultures were maintained for the next 48 hours in normal medium containing 10% FBS in the presence or absence of insoluble elastin (1 mg/well). Cell proliferation in 72 hour-cultures was first assessed with an inverted microscope with Nomarski optic and then cells were trypsinized and counted in a haemocytometer. Parallel sextiplicate cultures incubated
with the above mentioned agents were also exposed to \[^{3}\text{H}]\text{-thymidine (2 } \mu\text{Ci/well)}\] for the last 24 hours. These cultures were then washed in PBS and treated with cold trichloroacetic acid (TCA) twice for 10 minutes each time at 4°C. For the final 30 minutes 0.5ml of 0.3N NaOH was added to all dishes and 200 \(\mu\)l aliquots of each culture were then mixed with scintillation fluid and counted.

Statistics

In all biochemical studies, means and standard deviations were calculated, and statistical analyses were carried out by ANOVA.
Results

Costello syndrome fibroblasts produce extracellular matrix that lacks elastic fibers

Initial investigation into the fibroblasts from patients with Costello syndrome included a general survey of extracellular matrix (ECM) components. Using antibodies to specific components of the ECM, on 7-10 day old fibroblasts, immunocytochemical and quantitative analyses of elastin, fibrillin, MAGP, fibronectin and collagen type I were done. Quantitative summary of all the components from a morphometric analysis (fig. 5) and representative photomicrographs show the following: collagen type I and fibronectin both had higher than normal expression in fibroblasts from patients with Costello syndrome (fig. 7). Expression of microfibrillar scaffold proteins and cross-linking enzyme lysyl oxidase were comparable to normal fibroblasts (fig. 6). However, the expression of elastin was found to be much lower in Costello than in normal fibroblasts (fig. 8 B and 8A, respectively). These results were further reinforced by the lack of NaOH insoluble elastin present in the extracellular matrix produced by fibroblasts from patients with Costello syndrome (fig. 8C) measured by the assessment of [3H]-valine incorporation.

Costello syndrome fibroblasts produce normal amount of tropoelastin, but experience problems with its secretion and extracellular assembly

Quantitative assessment of [3H]-valine incorporation into immunoprecipitable soluble tropoelastin extracted jointly from the cell layers and from the conditioned media showed that fibroblasts from Costello syndrome patients and fibroblasts from normal children synthesized comparable amounts of tropoelastin (fig. 9A). The separate immunoprecipitation of metabolically labeled tropoelastin from the cell layer extracts and from the conditioned media clearly indicated that fibroblasts from Costello syndrome patients were characterized by an impaired secretion of newly synthesized tropoelastin (fig. 9B).
Figure 5

Morphometric analysis of extracellular matrix (ECM) components immunostained with specific antibodies in 10 day-old cultures of normal and Costello fibroblasts. Fibroblasts from Costello patients deposit only negligible amounts of immuno-detectable extracellular elastin. Amounts of fibronectin and collagen type I produced by Costello syndrome fibroblasts significantly exceed those present in cultures of normal fibroblasts. whereas deposition of fibrillin I and MAGP by Costello fibroblasts does not differ from normal fibroblasts. In each analyzed group, fifty low-power fields (x 20) from three separate cultures (per independent patients) were analyzed and the area occupied by the particular immunodetectable component quantified. The abundance of each component was then expressed as a percentage of the entire analyzed field (mean ± SD), and results from cultures of Costello syndrome fibroblasts were statistically compared with those in cultures of normal skin fibroblasts (* p< 0.001).
Morphometric Analysis of ECM Components Detected by Immunocytochemistry in 10 day-old Cultures of Fibroblasts

- Normal Fibroblasts
- Costello Fibroblasts

% of analyzed field occupied by ECM component

- Elastin
- MAGP
- Fibrillin I
- Collagen I
- Fibronectin

* indicates statistical significance.
Figure 6

Representative photomicrographs of 7-day-old cultures immunostained with anti-lysyl oxidase and anti-fibrillin type I. Expression of lysyl oxidase in Costello fibroblasts (B) does not differ from normal fibroblasts (A). Deposition of fibrillin I by Costello fibroblasts (D) does not differ from normal fibroblasts (C).
Figure 7

Representative photomicrographs of 7-day-old cultures immunostained with anti-collagen type I and anti-fibronectin. Costello fibroblasts deposit more collagen type I (B) and fibronectin (D) than normal fibroblasts (A and C, respectively).
Figure 8

Representative photomicrographs of 10-day-old cultures immunostained with anti-tropoelastin antibody indicate that normal fibroblasts (A) produced long, branching elastic fibers, while fibroblasts from Costello syndrome patient (B) did not deposit any extracellular elastin. (C) Quantitative analysis of tropoelastin indicates that Costello syndrome fibroblasts (7669, 9951, 10595) incorporate much less $[^3\text{H}]-\text{valine}$ into extracellular insoluble elastin than normal fibroblasts (3858, 4184, 4212). Fibroblasts from patients with Costello syndrome are characterized by a lack of extracellular insoluble elastin.
Figure 9

A - Quantitative analysis of $[^3\text{H}]-\text{valine}$ labeled immunoprecipitable tropoelastin indicates that both normal (3858, 4184, 4212) and Costello syndrome fibroblasts (7669, 9951, 10595) synthesize comparable amounts of total metabolically labeled tropoelastin.

B - In contrast to normal fibroblasts, Costello syndrome fibroblasts do not secrete the majority of their metabolically labeled tropoelastin.
In contrast to cultures of normal fibroblasts, in which cellular extracts contained only a small portion of total $[^{3}\text{H}]-\text{valine-labeled tropoelastin},$ the cell layer fractions of fibroblasts from Costello syndrome patients retained the majority of the metabolically labeled tropoelastin (fig. 10A). Moreover, SDS-PAGE and autoradiography of metabolically radiolabeled proteins immunoprecipitated with anti-tropoelastin antibody indicated that the bulk of soluble tropoelastin extracted from the fibroblasts of Costello syndrome patients was partially degraded. While cell extracts from normal children contained significant amounts of radiolabeled 70-kD tropoelastin, the cell layer extracts of fibroblasts from Costello syndrome patients demonstrated levels of intact 70-kD tropoelastin 3-5 times lower (as measured by densitometry), and showed numerous species of lower molecular weight representing tropoelastin degradation products (fig. 10B).

Costello syndrome fibroblasts produce normal EBP, but it is rapidly shed from their cell surface

Immunostaining with anti-S-GAL antibody, (which recognizes the elastin binding domain of EBP), consistently indicated that the levels of this protein were greatly diminished in fibroblasts from Costello syndrome patients. As depicted in Figure 11 A, EBP was particularly well expressed on the surface of normal fibroblasts. In contrast, the fibroblasts from Costello syndrome patients showed only negligible cell surface immunostaining (fig. 11B).

The metabolic pulse-chase labeling of EBP followed by the chase indicated that both types of fibroblasts initially synthesized comparable amounts of this protein. In contrast to normal fibroblasts, which demonstrated only a small decrease in labeled EBP during the chase period, the amounts of EBP extractable from fibroblasts of Costello...
Figure 10

A - Quantitative analysis of $[^3$H]-valine labeled tropoelastin indicates that Costello syndrome fibroblasts (7669.9951.10595) retain the majority of their metabolically labeled tropoelastin intracellularly compared to normal fibroblasts (3858.4184.4212).

B - Representative autoradiographs of metabolically radiolabeled proteins immunoprecipitated with anti-tropoelastin antibody from the cell layer extracts. In contrast to normal fibroblasts (3858.4184.4212) that contain a significant amounts of radiolabelled 70-kD tropoelastin, the Costello syndrome fibroblasts (7669.9951.10595) demonstrate much lower levels of intact 70-kD tropoelastin and show numerous lower molecular weight species likely representing tropoelastin degradation products.
A

Cell-associated Tropoelastin

Incorporation of $[^3H]$-valine CPM/μgDNA

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<th>Normal</th>
<th>Costello</th>
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<tr>
<td>3858</td>
<td>4184</td>
<td>4212</td>
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<td>7669</td>
<td>9951</td>
<td>10595</td>
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B

Immunoprecipitable tropoelastin

70-kDa

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<th>Normal</th>
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<td>3858</td>
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<td>7669</td>
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Figure 11
Representative photomicrographs of 48-hours-old fibroblast cultures lightly fixed in 100% cold methanol. Immunostaining with BCZ antibody, which recognizes EBP, indicates that normal fibroblasts (upper panel) demonstrate strong cell surface expression of this protein, while Costello syndrome fibroblasts (lower panel) show greatly diminished levels of the EBP.
CONTROL

COSTELLO
syndrome patients steadily decreased during the 45-minute chase (fig. 12, upper panel). The autoradiography did not detect any degradation products of EBP in the cell extracts from Costello syndrome and this metabolically labeled protein could be immunoprecipitated in increased amounts from the respective conditioned media during the chase, suggesting extensive shedding of EBP molecules from the cell surface of fibroblasts from Costello syndrome patients (fig. 12, lower panel).

Costello syndrome fibroblasts express intracellular and pericellular accumulation of chondroitin sulfate-bearing moieties

Another striking difference between fibroblasts from normal and Costello syndrome patients was their expression of the epitope detected with the monoclonal antibody raised to chondroitin sulfate A. Immunostaining of non-permeabilized cells showed that, in contrast to normal fibroblasts that deposited chondroitin sulfate extracellularly (fig. 13A), the fibroblasts from Costello syndrome patients were spotted with large amounts of immunogenic materials, likely representing accumulations of chondroitin sulfate-containing proteoglycans on their surface (fig. 13B). Immunostaining of permeabilized cultures indicated that in addition to extracellular matrix, the normal fibroblasts demonstrated the presence of chondroitin sulfate epitopes only in perinuclear regions (fig. 13C). Permeabilized fibroblasts from patients with Costello syndrome revealed accumulation of the chondroitin sulfate-containing material in multiple cytoplasmic vesicles (fig. 13D). Immunostaining of parallel cultures with antibody to the α subunit of hexosaminidase indicated that most of these chondroitin sulfate-positive vesicles could be identified as lysosomes. Additional immunohistochemical studies aimed at the positive identification of the chondroitin sulfate-bearing moieties accumulating in fibroblasts from Costello syndrome patients indicated that their intracellular vesicles (lysosomes) were also immunoreactive to antibodies recognizing the protein components of the chondroitin sulfate-bearing proteoglycans, CD-44 (fig. 14D) and biglycan (fig. 15D). In contrast to
Figure 12
Representative autoradiographs (upper panel) showing levels of the 67-kD EBP during the chase after metabolic pulse labeling with radioactive serine. Results indicate that fibroblasts from both normal and Costello syndrome patients initially synthesize comparable amounts of EBP, isolated by elastin affinity columns. In contrast to normal fibroblasts, which retain the majority of the labeled EBP, Costello syndrome fibroblasts steadily lose this newly produced protein, which can be immunoprecipitated in increased amounts from the respective conditioned media during the chase (lower panel).
Figure 13
Representative photomicrographs of 2-day-old cultures fixed with methanol (A-B) or with paraformaldehyde and then permeabilized with 0.2% Triton X-100 (C-D). Immunostaining with monoclonal antibody recognizing chondroitin sulfate indicates that in contrast to cultures of normal fibroblasts (A and C), which demonstrate immunodetectable epitope only in the extracellular matrix, Costello syndrome fibroblasts show strong cell surface staining associated expression of chondroitin sulfate (B). Fibroblasts from patients with Costello syndrome also demonstrate strong and overlapping intracellular (lysosomal) localization of chondroitin sulfate epitope in cultures permeabilized with Triton X-100 (D) compared to the absence of this staining in normal fibroblasts (C).
Figure 14

Representative photomicrographs of 2-day-old cultures fixed with methanol (A-B) or with paraformaldehyde and then permeabilized with 0.2% Triton X-100 (C-D). Immunostaining with hermes-1 antibody recognizing CD-44 showed similar cell surface staining in fibroblasts from Costello syndrome patients (B) and normal fibroblasts (A). Similar to staining with chondroitin sulfate antibody, CD-44 showed strong staining of intracellular vesicles in Costello syndrome fibroblasts (D) but showed very little staining intracellularly in normal fibroblasts (C).
Figure 15

Representative photomicrographs of 2-day-old cultures fixed with methanol. Immunostaining with biglycan antibody showed similar staining to chondroitin sulfate antibody, biglycan expression showed strong staining of intracellular vesicles in Costello syndrome fibroblasts (B and D) but did not show intracellular vesicularization in normal fibroblasts (A and C).
fibroblasts from Costello syndrome, normal fibroblasts did not reveal any storage of CD-44 (fig. 14 A and C) or biglycan (fig. 15 A and C). Interestingly, histochemistry with peanut agglutinin which selectively binds to Galβ1-3GalNAc domains showed identical patterns of cell surface and intracellular localization as anti-chondroitin sulfate antibodies (fig. 16).

Metabolic labeling of cultured fibroblasts with radioactive sulfate indicated that Costello syndrome fibroblasts accumulate much more sulfated proteoglycans than normal fibroblasts (fig. 17A). It has to be mentioned, however, that the amount of sulfated proteoglycans accumulated by Costello fibroblasts was considerably lower when compared with Hurler disease fibroblasts that store dermatan sulfate, lose EBP and display impaired elastogenesis (fig. 17B).

Enzymatic removal of chondroitin sulfate-bearing moieties from the cell surface improves elastogenesis in cultured Costello syndrome fibroblasts

Since the above mentioned results strongly indicated that impaired elastic fiber assembly in cultures of Costello syndrome fibroblasts coincides with accumulation of the immunodetectable chondroitin sulfate-bearing moieties, which induce shedding of EBP (Hinek et al 1988, 1991), we tested whether enzymatic removal of these galactosugar-bearing moieties from the cell surface would improve elastogenesis. Indeed, treatment of Costello syndrome fibroblast cultures with an enzyme that degrades chondroitin sulfates (chondroitinase ABC. 0.1 units/ml/ day, for ten days) led to restoration of normal elastic fiber assembly (fig. 18). In addition, co-culturing fibroblasts from patients with Costello syndrome in the presence of conditioned media from CHO cells transfected and capable of secreting enzymatically active precursor β-galactosidase into the conditioned media, also capable of partial GAG degradation, was able to partially restore elastic fiber assembly (Zhang et al 1994b).
Figure 16

Representative photomicrographs of 2-day-old cultures fixed with methanol (A-B) or with paraformaldehyde and then permeabilized with 0.2% Triton X-100 (C-D). Histochemistry with peanut agglutinin which selectively binds to Galβ1-3GalNac domains showed identical patterns of cell surface and intracellular localization as anti-chondroitin sulfate antibodies. Both methanol and paraformaldehyde fixation techniques showed the presence of intracellular vesicles in fibroblasts from patients with Costello syndrome (B and D). Normal fibroblasts showed extracellular fibrillar-like staining (A and C).
Figure 17

Metabolic labeling of cultured fibroblasts with radioactive sulfate for 7 days indicated that Costello syndrome fibroblasts accumulate much more sulfated proteoglycans than normal fibroblasts (p<0.01) (A). However, the amount of sulfated proteoglycans accumulated by Costello fibroblasts was considerably lower when compared with fibroblasts from patients with Hurler disease, a lysosomal storage disease that results in the storage of dermatan sulfate (B).
\[ 10.0 > d_\ast \]
Figure 18

Representative photomicrographs of 10-day-old cultures fixed with methanol. Fibroblasts from patients with Costello syndrome show an inability to assemble elastic fibers (A). Treatment with chondroitinase ABC, able to partially degrade chondroitin sulfate proteoglycans, was able to restore elastic fiber assembly (B).
Costello syndrome fibroblasts proliferate faster than their normal counterparts

In addition to the above-mentioned results, we have established that the growth rate of cultured fibroblasts from Costello syndrome patients was higher than normal fibroblasts. The striking difference in the cell density between normal and Costello syndrome fibroblasts was clearly noticeable in 3-day-old cultures (fig. 19). While normal fibroblasts initially plated at concentrations of 50,000 cells/well grew to an average 140,000 cells/well in the 3 days, the fibroblasts from Costello syndrome patients plated at the same initial density reached 320,000 cells/well (fig. 20A). Similar results indicating an increased rate of proliferation in cultures of fibroblasts from Costello syndrome patients were obtained when incorporation of \(^{3}\text{H}\)-thymidine was assessed (fig. 20B).

Exposure to exogenous insoluble elastin reverses the abnormally high proliferation of Costello syndrome fibroblasts

Interestingly, addition of exogenous insoluble elastin to cultures of fibroblasts derived from Costello syndrome patients significantly decreased their proliferation as assessed by inverted microscopy, by direct cell counting, and by incorporation of \(^{3}\text{H}\)-thymidine (fig. 19 and 20). Exogenous insoluble elastin not only caused inhibition of the cell growth, but also induced changes in the cell shape. While some Costello cells, which did not maintain direct contact with clusters of insoluble elastin remained elongated, cells attached to elastin clusters were spread and resembled normal fibroblasts. Treatment with insoluble elastin did not affect the growth rate of normal fibroblasts.
Figure 19

Representative phase-contrast micrographs of 3-day-old cultures illustrating that the growth rate of fibroblasts from Costello syndrome patients (B) was considerably higher than those taken from normal children (A). Addition of insoluble elastin, to cultures of Costello syndrome fibroblasts substantially reduced their cell density (C). Arrows indicate clusters of insoluble elastin attached to the cultured fibroblast.
Figure 20

Cell counting (A) and incorporation of $[^3\text{H}]$-thymidine (B) show the increased growth rate of Costello syndrome fibroblasts (solid bars) as compared to normal fibroblasts (open bars) in 3-day-old cultures. These assays also illustrate that high proliferation of Costello syndrome fibroblasts was significantly reduced in cultures treated with exogenous insoluble elastin (striated bars). Treatment with insoluble elastin did not change the proliferative rate of normal fibroblasts.

In all experiments cells were plated with the same initial density 50,000 cells/well. Values of mean ± SD from 3 different experiments were statistically compared with untreated controls within the same cell type (* $p<0.002$).
Discussion

Although the basic genetic defect in Costello syndrome remains obscure, the cardinal phenotypic features observed in Costello syndrome patients indicate that pathology of mesenchyme-derived tissues may be subjected to alterations contributing to problems in this clinical entity. Findings of defective elastic fibers in tissue samples from Costello syndrome (Vila Torres et al 1994; Mori et al 1996) reinforced this suggestion and focused our studies on the pathomechanism of impaired elastogenesis in Costello syndrome.

Results from the immunocytochemical and metabolic studies indicated that Costello syndrome fibroblasts demonstrated a normal rate of tropoelastin production, but were unable to secrete tropoelastin or to assemble extracellular insoluble elastin. The coincident finding of a very low immuno signal for EBP in Costello syndrome cells, initially suggested a deficiency in this recyclable tropoelastin chaperone (Hinek et al 1988, 1996; Hinek and Rabinovitch 1994) as a factor for the disruption of elastic fiber formation in Costello syndrome. The pulse-chase experiments indicated however, that fibroblasts from both normal and Costello syndrome patients initially produced equal amounts of the metabolically labeled 67-kD EBP, but only Costello syndrome fibroblasts quickly lost it into the conditioned media. We propose, therefore, that EBP is rapidly shed from Costello syndrome fibroblasts.

Since immunocytochemistry detected accumulation of the chondroitin sulfate-bearing moieties in Costello syndrome fibroblasts, we additionally suggest that shedding of EBP may occur upon contact of this protein with cell surface chondroitin sulfate moieties. This conclusion is based on our previous observations that binding of
galactosugar-containing moieties to the galactolectin domain of the EBP induces structural changes of this protein that make it unable to associate with tropoelastin and with the cell membranes (Hinek et al. 1988, 1993), and that addition of exogenous galacto-sugar bearing moieties (chondroitin sulfate or dermatan sulfate) to cultures of normal fibroblasts, chondroblasts and smooth muscle cells, caused depletion of EBP and disruption of elastic fiber production (Hinek et al. 1991, 1992). Decreased deposition of insoluble elastin by rat lung fibroblasts cultured in the presence of galactosamine-containing chondroitin sulfate and dermatan sulfate has also been reported by McGowan and colleagues (1993). Despite the fact that extracts of fibroblasts from Costello syndrome patients revealed the presence of degraded tropoelastin, cultures of these fibroblasts did not demonstrate any higher than normal activity of secretable elastolytic enzymes that could degrade exogenous insoluble elastin substrate. Thus, impaired formation of extracellular fibers, but not their accelerated degradation, is likely responsible for the low net content of insoluble elastin in the tissues of Costello patients. In fact, increased elastolysis would not be expected since chondroitin sulfate can sequestrate and inactivate leukocytic elastase (Volpi 1997; Ying et al. 1997).

Our proposal that the impaired elastogenesis observed in cells from Costello syndrome may be triggered by the accumulation of chondroitin sulfate-bearing moieties was reinforced by the fact that enzymatic degradation of these moieties by exogenous chondroitinase ABC or β-galactosidase led to restoration of normal elastic fiber assembly.

It should also be mentioned that results of our present study indicating links between impaired elastogenesis in Costello syndrome and functional deficiency of EBP induced by chondroitin sulfate-bearing moieties are consistent with our recent data of a very similar effect in Hurler disease, in which an accumulation of dermatan sulfate
(another galactosugar-bearing glycosaminoglycan) is associated with impaired elastogenesis (Hinek and Wilson 2000).


Our finding that permeabilized Costello syndrome fibroblasts display an apparent accumulation of the chondroitin sulfate-bearing proteoglycans (CD-44 and biglycan) in multiple cytoplasmic vesicles that overlap with immunolocalization of lysosomal enzyme, hexosaminidase further supports this claim.

We suggest that in Costello syndrome (similar to known mucopolysaccharidoses), the cells are not capable of normal degradation of the chondroitin sulfate attached to several different compounds, and that this inability may result in a storage of undegraded proteoglycans (CD-44, biglycan and others) in lysosomes. We speculate that a lack of a proper enzymatic degradation of chondroitin sulfate components of these proteoglycans may obscure their proteinase binding domains and consequently prevent further degradation of their protein cores. Despite the fact that a primary genetic defect in Costello syndrome is not caused by deficiency in α-L-iduronidase that triggers dermatan sulfate accumulation, and a consequent disruption of S-Gal-dependent elastic fiber assembly as in Hurler disease, it is quite possible that another enzyme involved in normal degradation of galactosugar-bearing moieties may be deficient in Costello syndrome. This possibility seems to be consistent with the recently reported suggestion concerning a
potential association of the Costello syndrome genetic error to chromosome 22 (Suri and Garrett 1998, van Eeghen 1999) that contains two copies of the gene for N-acetylgalactosamindase alpha, deficient in another mucopolysaccharidosis known as Schindler or alternatively Kanzaki syndrome (van Diggelen et al 1988). Further sequencing of genes in the 22q11 candidate region of the genome may eventually shed more light on the genetic locus for Costello syndrome.

Demonstration of impaired elastogenesis in Costello syndrome fibroblasts raises the important question whether this finding may be directly relevant to the clinical phenotypes observed in patients with this relatively new inherited disease.

Elastin is often thought to be a component produced in the latest stages of fetal development and in the perinatal period (Cleary et al 1967; Mecham and Hauser 1991; Parks et al 1993; Rosenbloom et al 1993; Pasquali-Ronchetti and Baccarani-Contri 1997). Recent studies with the developing chick embryo, using \textit{in situ} hybridization techniques revealed that tropoelastin mRNA is expressed during early development (Selmin et al 1991; Holzenberger et al 1993). Studies by Hurle and colleagues (1994) also demonstrated the presence of elastic fibers during early morphogenesis of the limb skeleton \textit{in vivo} and \textit{in vitro}, and suggested that the elastic fiber scaffold plays an important role in coordinating the size and the spatial location of the cartilaginous skeletal elements within the limb buds. They also observed precise patterns of elastic fiber arrangement present in the outflow tract and atrioventricular cushion tissue of the heart, the early developing lung, the notochord and the somites. These observations pointed to previously unsuspected functions for elastic matrices during embryonic development and substantiated our hypothesis, that impaired elastogenesis in the developing skeleton, heart or skin may be play an important role in the overall pathophysiology of the Costello syndrome.
Our observation that fibroblasts from Costello syndrome patients, which are unable to assemble normal elastic fibers, displayed an increased rate of proliferation as compared to normal skin fibroblasts indicates a potential pathophysiological link between the absence of insoluble elastin and increased cell proliferation. Further support for this link comes from a recent observation of Li and colleagues (1998) who showed that newborn transgenic mice lacking elastin protein die as a result of arterial blockage with abnormal intimal thickenings comprised of proliferating smooth muscle cells.

The possibility that insoluble elastin may, in fact, negatively modulate mitogenic signals is further substantiated by the fact that administration of exogenous insoluble elastin to cultured Costello syndrome fibroblasts normalized their proliferation rate. Increased cellular proliferation associated with decreased insoluble elastin in extracellular matrix produced by Costello syndrome fibroblasts may result from several overlapping mechanisms. Firstly, large hydrophobic particles of exogenous insoluble elastin may attract soluble tropoelastin-derived peptides present in the conditioned media and cause their precipitation (coacervation). Such a depletion of soluble fragments of tropoelastin, known to be stimulators of cell cycle progression (Jung et al 1998; Hinek et al 1999) may therefore down-regulate cellular proliferation. Secondarily, establishment of physical contact between large particles of insoluble elastin and the cell surface may cause binding and trapping of EBP molecules that prevent their shedding. Aggregation of cell surface EBP may, in turn, lead to masking of adjacent growth factor receptors that normally transduce mitogenic signals induced by serum-derived growth factors. Conversely, extensive chondroitin sulfate-induced shedding of EBP from the cell surface of Costello syndrome fibroblasts may lead to unmasking of adjacent cell surface receptors important for mitogenic signal transduction. This suggestion is consistent with our previously reported results showing that chondroitin sulfate-dependent shedding of EBP from cell surfaces of arterial smooth muscle cells unmasks their adjacent interleukin type I receptors interacting with IL-1β and facilitates cellular response to this cytokine (Hinek
et al 1996). This results in a net increase in fibronectin production (also noted in cultures of Costello syndrome fibroblasts) and stimulation of cell migration and proliferation.

This proposed mechanism does not preclude the possibility that chondroitin sulfate-containing proteoglycans accumulating on the surface of fibroblasts from Costello syndrome patients may also act as co-receptors for major growth factors (e.g., bFGF). In this regard, our finding of increased expression of CD44 in fibroblasts from Costello syndrome patients may be of special interest. This cell surface hyaluronate receptor containing a chondroitin sulfate moiety, which may act as a low affinity fibroblast growth factor receptor, has been widely implicated in the growth of numerous human tumors (Borlund et al 1998; Ponta et al 1998; Herrlich et al 1998; Sneath and Mangham 1998; Chiu et al 1999; Humphrey et al 1999), including rhabdomyosarcoma which has been reported in Costello syndrome patients (Kerr et al 1998; Feingold 1999).

In summary, results presented in this thesis indicate that:

- Chondroitin sulfate-dependent shedding of EBP from fibroblasts of Costello syndrome patients eventually eliminates recycling of this tropoelastin chaperone, and consequently disrupts tropoelastin secretion and extracellular assembly into elastic fibers.

- Costello syndrome should be added to the list of inherited diseases which are characterized by impaired elastogenesis.

- Lack of insoluble elastin coincident with high rates of cell proliferation may be relevant to the pathophysiological mechanisms responsible for the development of the cardinal phenotypic features of the Costello syndrome, including skeletal, cardiovascular and skin problems, as well as the development of benign and malignant tumors.
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