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Type IX Ehlers-Danlos Syndrome and Menkes Syndrome: The Decrease in Lysyl Oxidase Activity Is Associated with a Corresponding Deficiency in the Enzyme Protein

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SUMMARY

Type IX of the Ehlers-Danlos syndrome (E-D IX) and the Menkes syndrome are X-linked recessively inherited disorders characterized by abnormalities in copper metabolism. These abnormalities are associated with a severe reduction in the activity of lysyl oxidase, the extracellular copper enzyme that initiates crosslinking of collagens and elastin. No increase in this deficient enzyme activity was obtained when culture media from fibroblasts of patients with E-D IX or the Menkes syndrome were incubated with copper under various conditions in vitro. A distinct, although small, increase in lysyl oxidase activity was obtained, however, when copper-supplemented media were used during culturing of the fibroblasts, although even under these conditions, the enzyme activity in the media from the affected cells remained markedly below that of the controls. Immunoprecipitation, dot-blotting, and immunoperoxidase staining experiments with antisera to human lysyl oxidase indicated that fibroblasts from patients with E-D IX or the Menkes syndrome do not secrete into their medium, or contain inside the cell, any significant amounts of a copper-deficient, catalytically inactive lysyl oxidase protein. These findings appear to be consistent with the hypothesis that synthesis of the lysyl oxidase protein itself is impaired. The possibility is not excluded, however, that a copper-deficient enzyme protein may be synthesized in normal amounts but become degraded very rapidly inside the cell. The failure to obtain any large increase in the deficient lysyl oxidase activity upon various forms of copper administration suggests that it may not be possible to obtain any significant improvement in

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the connective tissue manifestations of these disorders by copper therapy.

INTRODUCTION

Type IX of the Ehlers-Danlos syndrome (E-D, McKusick 30415 in [1]) and the Menkes steely hair syndrome (McKusick 30940) are X-linked recessively inherited disorders characterized by alterations in copper metabolism [1–5]. The clinical manifestations of E-D IX include bladder diverticula with spontaneous ruptures, inguinal hernias, slight skin laxity and hyperextensibility, and a number of skeletal abnormalities, a peculiar feature being occipital horn-like exostoses [1, 2, 4–9]. The Menkes syndrome also includes bladder diverticula, mildly increased skin laxity and hyperextensibility, and skeletal abnormalities, but unlike E-D IX, it also involves arterial disease and neurological changes so severe that the disorder usually proves lethal by 3 years of age [1–5]. Several very similar abnormalities in copper metabolism are apparent in these two disorders. Serum copper and ceruloplasmin concentrations are low, whereas cultured fibroblasts have markedly elevated concentrations of the cation [1–5, 8, 10, 11]. In both disorders, the excessive copper accumulates in the cation-binding protein metallothionein or a metallothionein-like protein that is present in cultured fibroblasts in highly increased amounts [1–5, 10, 11]. The induction of metallothionein may not be the primary abnormality, however, but may be secondary to some unidentified primary defect in cellular copper transport or utilization [1–5, 10–12].

All the connective tissue manifestations in these two disorders are probably due to a reduction in the activity of lysyl oxidase, the extracellular copper enzyme that initiates crosslinking in collagen and elastin [13]. A deficiency in this enzyme activity has been demonstrated in skin specimens [6, 10] and in the medium of cultured fibroblasts [6, 8, 10] from patients with E-D IX and in the medium of cultured fibroblasts from patients with the Menkes syndrome [10, 14, 15]. The mechanisms leading to this deficiency are unknown, however. A high negative correlation has been demonstrated in these two disorders between the cellular copper concentration and the logarithm of lysyl oxidase activity, suggesting a distinct relation between these alterations [10]. Thus, the deficient enzyme activity may be secondary to a decrease in functional intracellular copper, even though the cellular concentrations are highly increased [15]. The possibility is not excluded, however, that the genes coding for lysyl oxidase, some other copper-dependent enzymes, and a cellular copper transport protein may be linked by some mechanism such that a single mutation will affect the function of all of them to varying degrees [16]. In this case, there would be a decrease in the synthesis of the lysyl oxidase protein itself.

Here, we studied whether it would be possible to obtain an increase in lysyl oxidase activity in E-D IX and the Menkes syndrome either by incubating culture media from the affected cells with copper in vitro or by administering

varying concentrations of copper to cells in culture. We also examined whether a copper-deficient, catalytically inactive enzyme protein is present in these two disorders, either in the culture medium or inside the cells.

MATERIALS AND METHODS

Cell Cultures

Fibroblast cultures for the E-D IX family were established from skin biopsies from two patients aged 24 (E-D IX 1) and 13 years (E-D IX 2) whose clinical manifestations and abnormalities in copper and collagen metabolism have been described [8–10]. The Menkes fibroblast cultures were established by Dr. N. Horn at the Department of Medical Genetics, John F. Kennedy Institute, Glostrup, Denmark, from skin biopsies from 11 definite, typical patients, numbered Menkes 1-11 according to the severity of the abnormality in radioactive copper incorporation [10]. Menkes 1 had the most severe degree of abnormality and Menkes 11 the mildest [10]. Control fibroblast cultures were established from skin specimens from apparently healthy subjects that corresponded to the E-D IX or Menkes patients in age.

The cells were grown in 65-cm^2 plastic culture dishes (Nunc, Roskilde, Denmark) in 10 ml of Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 290 μg/ml L-glutamine. $CuSO_4$ and other substances were added to some of the cultures as indicated in the description of the individual experiments under RESULTS. For the measurement of lysyl oxidase activity, fibroblasts that had just reached confluent density were washed twice with a solution of 10 mM sodium phosphate and 0.14 M NaCl, pH 7.4, and were then cultured for 24 hrs in 5 ml of the above medium without serum but supplemented with 5 mg/ml of bovine serum albumin (corresponding to an albumin concentration of 10% serum). In the experiments involving dot-blotting, the fibroblasts were grown for the last 24 hrs with either 10% serum or 5 mg/ml bovine serum albumin, as the results obtained under these two conditions were identical. All media contained 50 μg/ml ascorbate.

Antisera

Two specific antisera to human placental lysyl oxidase were available, one an antiserum to lysyl oxidase purified from the DEAE-cellulose chromatography peaks 1 and 2 (antiserum I) and the other (antiserum T) to total lysyl oxidase protein, that is, DEAE-cellulose chromatography peaks 1–4 [17]. Both were immunoadsorbed on a column containing a purified lysyl oxidase contaminant linked to agarose [17]. Antiserum to human prolyl 4-hydroxylase had been prepared as described [18].

Immunoprecipitation, Dot-blotting, and Immunostaining

Lysyl oxidase was immunoprecipitated from culture media containing bovine serum albumin with varying amounts of antiserum T as described [17], and aliquots of the supernatant were assayed for enzyme activity. Dot-blotting was carried out by dotting varying amounts of the culture media onto nitrocellulose sheets (Schleicher & Schüll, West Germany) with a micro-capillary pipette, 2 μl at a time [19]. The sheets were air-dried, and unspecific protein binding was blocked by incubation for 2 hrs at 22°C in a buffer with a high protein content [20]. The nitrocellulose sheets were then incubated overnight at 22°C with either immunoadsorbed antiserum I diluted 1:40 with the high protein buffer [20] or immunoadsorbed antiserum T diluted 1:80. After repeated washings with the high protein buffer, immunostaining was performed using a peroxidase-antiperoxidase complex technique [21].

For immunohistochemical staining of the enzyme proteins in the cultured fibroblasts, the cells were cultured on coverslips that were placed on tissue culture dishes. Slips were removed just as the cells reached confluent density, rinsed three times with a

solution of 10 mM sodium phosphate and 0.14 M NaCl, pH 7.4, fixed for 2 min with either ice-cold methanol or ice-cold 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and rinsed three times for 5 min with a solution of 10 mM sodium phosphate and 0.14 M NaCl, pH 7.4. The immunostaining was performed using either immunoadsorbed lysyl oxidase antiserum I diluted 1:10, immunoadsorbed antiserum T diluted 1:20, or prolyl 4-hydroxylase antiserum diluted 1:80. The antigen-antibody complexes were detected using a peroxidase-antiperoxidase complex technique [21]. All the immunostaining reagents were purchased from Dakopatts (Glostrup, Denmark).

DEAE-Cellulose Chromatography of Lysyl Oxidase

Media from the cultured fibroblasts, to a volume of 60 ml, were dialyzed four times against 1,000 ml of 6 M urea in 10 mM sodium phosphate, pH 7.8, at 4°C for 4 days, and 50 ml of the dialyzed medium was applied to a 30-ml DEAE-cellulose column equilibrated with the same urea buffer. The column was washed with 30 ml of the above solution and then eluted with a 40-ml linear gradient of 0–0.5 M NaCl in the urea buffer, followed by 40 ml of 0.5 M NaCl in the same buffer. Fractions of about 1 ml were collected and dialyzed twice against 5,000 ml of 0.15 M NaCl in 0.1 M sodium phosphate, and aliquots of the dialyzed fractions used to assay lysyl oxidase activity.

Assay of Lysyl Oxidase Activity

The enzyme activity was measured in a final volume of 0.6 ml with 0.8×10^6 dpm of [6^3H]lysine-labeled purified chick-embryo calvaria collagen substrate [22]. The substrate was preincubated at 37°C for 60 min to promote fibril formation [22], and the incubation time with the enzyme was then 10 hrs [10]. The substrate was prepared as described [10, 17]. The cell culture media, which had been stored frozen at −20°C for up to 1 month, were dialyzed for 3 hrs at 4°C against a solution of 0.15 M NaCl in 0.1 M sodium phosphate, pH 7.8, and aliquots of 0.25 and 0.50 ml were then used for the assays without any prior concentration [10]. As reported [10], about 90% of the total lysyl oxidase activity in all the fibroblast types studied here was in the culture medium. The remaining 10% can be extracted from the cell layers with 4 M urea in all fibroblast types [10], but, except for the immunohistochemical staining experiments, no further studies were performed on this activity.

RESULTS

Attempts to Stimulate Lysyl Oxidase Activity by Cu Supplementation

Attempts were made in preliminary experiments to stimulate the low lysyl oxidase activity found in the medium of cultured E-D IX and Menkes fibroblasts by adding 5–100 μM Cu to the enzyme assay mixture. No stimulation was found in any of the experiments with control, E-D IX, or Menkes fibroblasts when the media containing bovine serum albumin (see MATERIALS AND METHODS) were assayed in the presence of several different $CuSO_4$ concentrations. Neither was any stimulation found in experiments in which the culture media were assayed in the presence of 0.5 mg/ml of ceruloplasmin or 0.5 mg/ml of ceruloplasmin and 5–50 μM $CuSO_4$ (details not shown).

In the subsequent experiments, $CuSO_4$ was added to the medium containing bovine serum albumin for the last 24 hrs of fibroblast culture. A slight increase in lysyl oxidase activity was consistently found in these experiments, the highest degree of stimulation being found with 50 μM Cu (experiments 1 and 2 in table 1). This Cu concentration is about 50 times that present in the nonsupple-

TABLE 1

STIMULATION OF LYSYL OXIDASE ACTIVITY IN THE MEDIUM OF CULTURED FIBROBLASTS BY SUPPLEMENTATION WITH EITHER 50 μM Cu FOR 24 HRS (EXPERIMENTS 1 AND 2) OR 8 μM Cu FOR 96 HRS (EXPERIMENT 3)

CULTURES	LYSYL OXIDASE IN dpm/10^6 CELLS*		
	Without added Cu (A)	With added Cu (B)	B/A
	50 μM, 24 hrs		
Experiment 1†:			
Control 1	6,340	8,340	1.32
E-D IX 1	990	1,730	1.75
	50 μM, 24 hrs		
Experiment 2†:			
Control 2	6,580	7,790	1.18
Control 3	6,990	8,550	1.22
E-D IX 1	1,000	1,830	1.83
	8 μM, 96 hrs		
Experiment 3‡:			
Control 5	6,500	6,930	1.07
Control 6	7,070	7,360	1.04
E-D IX 1	1,330	2,120	1.59
Menkes 8	1,610	2,730	1.70

NOTE: E-D IX 1 refers to patient 1 with E-D IX, and Menkes 8, to patient 8 with the Menkes syndrome.

* Each value shown is the mean of duplicate assays.

† Fibroblasts just reaching confluency were cultured for 24 hrs in 5 ml of the serum-free medium supplemented with 5 mg/ml bovine serum albumin (see MATERIALS AND METHODS), with or without an addition of 50 μM $CuSO_4$. Lysyl oxidase activity was then assayed in aliquots of this medium.

‡ Half of the fibroblast cultures received 8 μM $CuSO_4$ in the medium supplemented with 10% fetal calf serum about 3 days before reaching confluency and were cultured further for 72 hrs. The cells were then grown for an additional 24 hrs in the serum-free medium containing 5 mg/ml bovine serum albumin, with or without an addition of 8 μM $CuSO_4$. Lysyl oxidase activity was assayed in aliquots of this medium.

mented culture medium, as determined by atomic absorption spectrophotometry. The stimulation found with the E-D IX cells (experiments 1 and 2 in table 1) or Menkes cells (not shown) was consistently slightly greater than that found with the control cells. Nevertheless, as no further stimulation was found with higher Cu concentrations, the values for the affected cells remained markedly below those of the controls even in the presence of Cu supplementation.

In further experiments, the cells were cultured with added $CuSO_4$ for the last 96 hrs, and the media containing bovine serum albumin collected during the last 24 hrs were assayed for lysyl oxidase activity. Under these conditions, 8 μM Cu (about eight times the concentration in the nonsupplemented culture medium) gave maximal stimulation (one example shown as experiment 3 in table 1), no further increase being obtained with higher concentrations. The stimulation found with the control cells was less than 1.2-fold in all the experiments, whereas that found with the affected cells ranged from about 1.5- to 2.0-

fold. The addition of 0.5 mg/ml of ceruloplasmin or 0.2 μg/ml of glycyl-histidyl-lysine, substances that bind Cu and might thus help the transport of this cation [23, 24], did not increase the stimulation obtained with $CuSO_4$ any further.

Immunoprecipitation of Lysyl Oxidase Activity from the Medium of Cultured Fibroblasts by a Specific Antiserum

To study whether the E-D IX and Menkes fibroblasts secrete inactive lysyl oxidase protein into their medium, the enzyme activity was precipitated from the medium of cultured fibroblasts using an antiserum prepared to human placental lysyl oxidase [17]. Of the two slightly different preparations, antiserum T was more effective in precipitating lysyl oxidase activity [17], whereas antiserum I was more effective in staining the denatured enzyme protein during immunoblotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis [17]. Antiserum T was used in the precipitation experiments.

The precipitation curves obtained were similar to those reported [17], and a linear relationship was found between the number of enzyme activity units added and the amount of antiserum required to precipitate 50% of the enzyme activity. The latter amount was markedly lower in the culture media from the E-D IX and Menkes cells than in those from the controls, when expressed per 10^6 cells (table 2). However, when expressed per 1,000 dpm of lysyl oxidase activity, the values for all three cell types were essentially identical (table 2). The E-D IX and Menkes fibroblasts therefore do not appear to secrete any inactive lysyl oxidase protein into their medium that would compete with the active enzyme for binding to the antiserum.

Assay of Immunoreactive Lysyl Oxidase in the Culture Media by Dot-blotting

The culture media from E-D IX and Menkes fibroblasts were also seen to contain markedly lower amounts of immunoreactive lysyl oxidase protein than those from the control fibroblasts when studied by dot-blotting with antiserum I

TABLE 2

PRECIPITATION OF LYSYL OXIDASE ACTIVITY FROM THE MEDIUM OF CULTURED FIBROBLASTS BY A SPECIFIC ANTISERUM

CULTURES	LYSYL OXIDASE ACTIVITY dpm/10^6 cells	ANTISERUM REQUIRED FOR 50% PRECIPITATION	
		μl/10^6 cells	μl/1,000 dpm
Control 1	6,210	27.2	4.38
Control 2	6,110	25.6	4.19
E-D IX 1	1,460	6.15	4.21
Menkes 2	470	2.14	4.56
Menkes 3	900	3.67	4.08

NOTE: Lysyl oxidase was immunoprecipitated from the culture media with varying concentrations of antiserum T prepared to human placental lysyl oxidase [17]. The volume of antiserum required for precipitation of 50% of the enzyme activity was then determined from the concentration curves obtained, these curves being similar to those shown previously [17]. E-D IX 1 refers to patient 1 with E-D IX, and Menkes 2 and 3 refer to patients 2 and 3 with the Menkes syndrome.

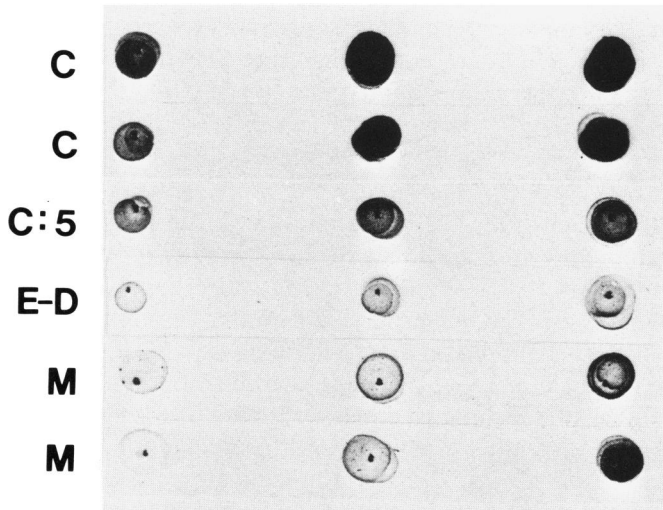

FIG. 1.—Assay of immunoreactive lysyl oxidase protein by dot-blotting. Varying amounts of media from confluent cultures of control (*C*), E-D IX (patient *E-D* 1), and Menkes (patients *M* 2 and 3) fibroblasts were dotted onto nitrocellulose sheets and stained for immunoreactive lysyl oxidase with antiserum I as described under MATERIALS AND METHODS. One of the two control media were also studied in a 1:5 dilution (*C:5*). The volumes of the media dotted were 2 μl, 6 μl, and 10 μl.

(fig. 1) or antiserum T (not shown). The enzyme activity in the media from the E-D IX and Menkes fibroblasts shown in figure 1 was about 5%–20% of that in the medium from the controls shown. The amount of immunoreactive enzyme protein would seem to be even slightly more reduced (fig. 1) in some of the dots, but this discrepancy is due to some nonlinearity in the staining when the enzyme concentrations are very low.

Staining of Cultured Fibroblasts with Antisera to Lysyl Oxidase by Immunoperoxidase Techniques

Since the immunoprecipitation and dot-blotting experiments suggested that E-D IX and Menkes fibroblasts do not secrete any inactive lysyl oxidase protein into their culture medium, attempts were made to study whether such inactive enzyme protein accumulates within the cells. Staining of the cells with antiserum T (fig. 2) or antiserum I (not shown) by immunoperoxidase techniques indicated that the immunoreactive enzyme concentration in the E-D IX and Menkes cells was not increased but, instead, markedly decreased (fig. 2). Control experiments with an antiserum to human prolyl 4-hydroxylase, an intracellular enzyme of collagen synthesis [18], showed no difference in staining among the three cell types (fig. 2).

Characterization of Lysyl Oxidase Activity in the Culture Medium by DEAE-Cellulose Chromatography

Preparations of lysyl oxidase from several sources are heterogeneous, with multiple peaks of activity in DEAE-cellulose chromatography, but identical

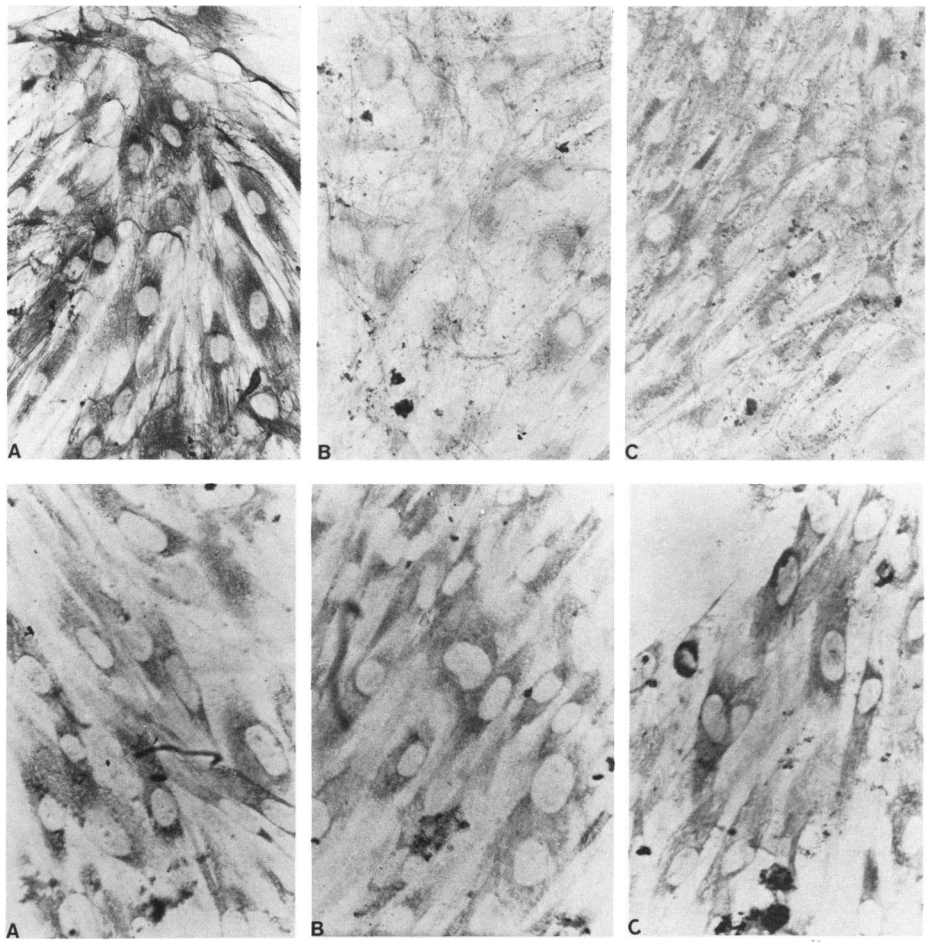

FIG. 2.—Staining of cultured fibroblasts with antiserum to lysyl oxidase by immunoperoxidase techniques. The fibroblasts, cultured on cover slips, were stained either with antiserum T to lysyl oxidase (*upper panel*) or antiserum to human prolyl 4-hydroxylase (*lower panel*). *A*, control fibroblasts; *B*, E-D IX fibroblasts (patient 1); and *C*, Menkes fibroblasts (patient 3). No counterstaining of nuclei was performed. Original magnification 110×.

molecular weight [13, 17, 25]. The differences may exist at the level of some post-translational modification of the lysyl oxidase protein, but the possibility is not excluded that the multiple enzyme forms represent products of several very similar but not identical genes [17, 25]. In the latter case, they could have different affinities for Cu, and thus the pattern of lysyl oxidase activity peaks seen in DEAE-cellulose chromatography of the media from E-D IX and Menkes cells might be altered, with one or two of the activity peaks predominating.

In agreement with data on other sources, the lysyl oxidase in the medium of the control fibroblasts showed multiple peaks of activity in DEAE-cellulose chromatography (fig. 3). All activity peaks were reduced in size in the media

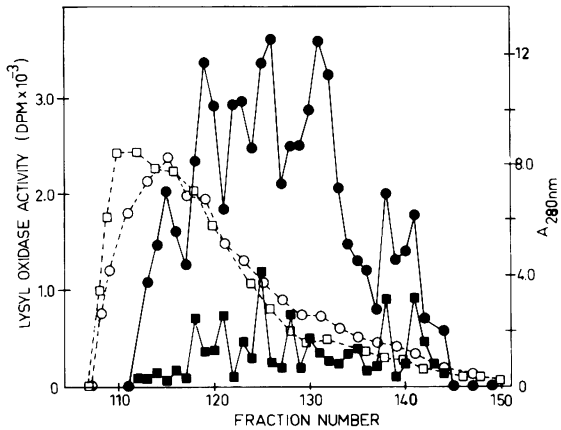

FIG. 3.—DEAE-cellulose chromatography of fibroblast medium from the control and E-D IX cells. The chromatographies were carried out as described under MATERIALS AND METHODS. Only the elution with the gradient of 0–0.5 M NaCl in 6 M urea and 10 mM sodium phosphate, pH 7.8, is shown. Lysyl oxidase activity is given as dpm/fraction for the control (●) and E-D IX (■) medium. A_{280} nm of the control (○) and E-D IX (□) medium is also shown.

from E-D IX (fig. 3) and Menkes (not shown) fibroblasts, and, thus, there was no evidence for a less-marked decrease in the size of one or two of the peaks. The activity values found for the individual DEAE-cellulose column fractions in the media from the affected cells were too low to be assayed accurately, and therefore the peak pattern found for the affected cells is somewhat different from that found for the controls.

DISCUSSION

Cultured fibroblasts from patients with E-D IX or the Menkes syndrome do not appear to secrete into their medium or contain inside the cell any significant amounts of copper-deficient, catalytically inactive lysyl oxidase protein. This conclusion is based on immunoprecipitation, dot-blotting, and immunoperoxidase staining results obtained with two different antisera, both of which recognize the native and denatured forms of the enzyme [17]. Although one of the antisera is more effective in precipitating the active enzyme while the other is more effective in staining the denatured enzyme protein in immunoblotting [17], the uniform results obtained with both strongly suggest that the failure to detect such an inactive enzyme is not due to any methodological artifact. These data would be consistent with the hypothesis [15] that synthesis of the lysyl oxidase protein itself is impaired. The possibility is not excluded, however, that a copper-deficient enzyme protein may be synthesized in normal amounts but become degraded very rapidly inside the cell. Further elucidation of these aspects would greatly benefit from lysyl oxidase cDNA probes and measurements of the enzyme mRNA concentrations.

No significant differences in catalytic, molecular, or immunological properties have been found previously between the multiple lysyl oxidase forms [13,

17, 25]. Nor are any differences indicated here between the multiple enzyme forms in the case of the deficient lysyl oxidase activity in E-D IX or the Menkes syndrome, as DEAE-cellulose chromatography did not demonstrate any predominance of one or two of the enzyme activity peaks in the culture media from the affected fibroblasts.

The absence of copper-deficient inactive lysyl oxidase protein in the culture media from E-D IX and Menkes fibroblasts explains the fact that it was not possible to obtain any increase in enzyme activity by incubation of the culture media with copper under various conditions in vitro. If a similar deficiency in lysyl oxidase protein is also present in vivo, it is evident that no therapeutic procedures will increase the enzyme activity simply by activating an inactive enzyme protein. A distinct, although small, increase in lysyl oxidase activity was obtained, however, when copper-supplemented media were used during culturing of the cells. Nevertheless, this increase was only slightly larger in the E-D IX and Menkes fibroblasts than in the control cells, leaving the enzyme activity of the affected fibroblasts markedly below the control values even in the presence of optimal Cu concentrations. These data agree with reports on the effect of subcutaneous copper therapy in polyether/sebacic acid solution to Blotchy mice [26] featuring connective tissue manifestations due to similar alterations in copper metabolism and lysyl oxidase activity as were found in the patients studied here [3, 16, 26]. The administration of copper to the Blotchy mice increased their lysyl oxidase activity, which was originally about 20% of the control mean, only about 1.6-fold, and thus the value remained very low [26]. Brindled mice also have similar alterations in copper metabolism, but no connective tissue manifestations and only mildly reduced lysyl oxidase activity, about 50%–70% of that in their littermate controls [16, 26]. In these mice, it is possible to increase the enzyme activity to the same level as in the nontreated controls by similar subcutaneous copper therapy [26]. In the light of these studies on mice mutants and the present data, it seems that it may not be possible to remedy the low lysyl oxidase activity in patients with E-D IX and the Menkes syndrome to any therapeutically significant extent by parenteral copper administration.

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