LYSYL OXIDASE DEFICIENCY IN LUNG AND FIBROBLASTS FROM MICE WITH HEREDITARY EMPHYSEMA

Barry C. Starcher, Judy A. Madaras and Arnold S. Tepper

Washington University Medical Center, Department of Internal Medicine, Pulmonary Disease Division, 660 South Euclid, St. Louis, Missouri 63110

Received August 18,1977

SUMMARY

Lysyl oxidase activity was measured in the lungs and from cultured fibroblasts of Blotchy mice. A marked decrease in lysyl oxidase activity was observed in lungs of affected mice as compared to normal litter mates. Fibroblasts cultured from Blotchy mice were also deficient in lysyl oxidase, producing less than half of normal enzyme levels. Normal and Blotchy fibroblasts which had been maintained in culture for several months and had undergone spontaneous transformation, continued to show the same magnitude of difference in lysyl oxidase levels. The data suggest that the deficiency of lysyl oxidase is inherent in Blotchy fibroblasts and support the idea that the deficiency of this enzyme is the metabolic lesion that leads to the connective tissue defects observed in these animals.

INTRODUCTION

Mice with "Blotchy" alleles at the X chromosomal locus have decreased levels of specific lysine derived aldehydes (allysines) involved in the cross-linking collagen and elastin (1). These animals exhibit connective tissue abnormalities similar to those found in lathrytic animals, including bony deformities, reduced tensile strength of skin, and aortic aneurysms (2). Fisk and Kuhn (3) reported that Blotchy mice have defective lung elastin and changes in lung structure that resemble panacinar emphysema.

Lysyl oxidase is the enzyme responsible for the initial oxidative step in allysine formation. Rowe et al postulated a deficiency of lysyl oxidase in Blotchy mice several years ago and recently have reported significantly reduced levels of this enzyme in the skin of Blotchy mice (4). In the present study we have found a decreased number of desmosine cross-links and a marked decrease in the lysyl oxidase activity in Blotchy mouse lungs as compared to those of normal mice. We have also investigated the lysyl oxidase activity released by fibroblasts in culture and found a significant decrease in the enzyme activity of the Blotchy as compared to normal cells.

ISSN 0006-291X

METHODS

Fibroblasts were grown from stomach muscle explants of abdominal wall of 11 day old Blotchy and normal mice. The tissue was minced into approximately 1 mm^2 sections and placed in 25 cm^2 plastic flasks containing 5 ml Dulbecco's modified Eagle medium (Kansas City Biologicals) (20% fetal calf serum) with 150 units/ml of penicillin, and 160 µg/ml of streptomycin. The cultures were incubated at 37° in 5% CO_2 - 95% oxygen atmosphere and the medium was renewed biweekly. The first outgrowths of fibroblasts occurred between three to five days after culturing. From three weeks to one month later approximately 1.5 million cells were ready for subculture from each flask. To subculture, the media was removed from the cells, and the cells were first rinsed in Hanks (Kansas City Biologicals) balanced salt solution, and subsequently incubated in 0.1% trypsin (2X crystallized) for 10 minutes at 37° C. The cells were removed in trypsin, and centrifuged for 10 minutes at 1000 rpm after the addition of fresh medium. The cell pellet was then resuspended in fresh medium, counted, and $2x10^5$ cells/plate dispensed into 60x15 mm tissue culture dishes. The media was removed every 48 hours and assayed for lysyl oxidase. Protein was determined by the method of Lowry (5).

Lysyl oxidase was determined using the method of Pinnell and Martin (6) except for changes in the substrate. The modified substrate was prepared by culturing 4 gm of finely minced fetal calf ligamentum nuchae in a 200 ml Erlenmeyer flask containing 40 ml of lysine deficient Dulbecco's modified Eagle's medium (Biolabs, Northbrook, IL) supplemented with 2000 iµ penicillin and streptomycin, 2 mg β-aminopropionitrile, 2 mg ascorbic acid and 200 µCi 4-5-^{3}H-D,L-lysine. The flasks were incubated in 5% CO_2 - 95% oxygen atmosphere for 24 hours. After the incubation the ligament was washed thoroughly with water and homogenized in 0.01 M NaH_2PO_4 buffer, pH 7.5 containing 0.15 M NaCl and 4 M in urea. The homogenate was centrifuged at 18,000 g for 30 minutes, the supernatant was removed and the pellet resuspended in buffer and the urea extraction was repeated. The combined supernatants were dialyzed against distilled water. The precipitate which formed was removed by centrifugation and the supernatant lyophilized and stored at -20° C until used in the assay. Typical yield of substrate from 4 gm of ligament was 100 mg with a specific activity of 150,000 cpm/mg. In preliminary studies we found the substrate was partially destroyed by both elastase and collagenase, suggesting that the substrate is composed of both elastin and collagen.

For the assay of lysyl oxidase from mouse lung the whole lung was removed from animals approximately 4 months of age and homogenized in 0.1 M phosphate buffer, pH 7.7. The homogenate was centrifuged, the supernatant removed and the precipitate resuspended in 4 ml of phosphate buffer containing 4 M urea. After extraction for 4 hours at 4° C the suspension was centrifuged and the supernatant decanted and dialyzed for 24 hours against several changes of 0.05 M NaH_2PO_4, 0.15 M NaCl, buffer pH 7.7. The lysyl oxidase assay consisted of incubating 0.2 ml of the enzyme solution with 0.5 mg substrate and 0.8 ml of pH 7.7 phosphate buffer for 4 hours at 37° C. The tritiated water formed was collected by distillation and quantitated by liquid scintillation spectrometry. Enzyme activity was determined as the difference in tritium released in the presence of enzyme and a blank containing the enzyme plus 50 µg/ml of β-aminopropionitrile.

Measurements of lysyl oxidase from 5 ml of culture media was accomplished by concentrating the media to 1 ml in a macrosolute concentrator (Minicon B-15, Amicon). The enzyme concentrate (0.5 ml) was mixed with 0.1 ml of the substrate containing 75,000 cmp and 1 ml phosphate buffer. After a 4 hour incubation at 37° C the tritiated water was distilled and counted.

Desmosines were quantitated in normal and Blotchy lungs as described previously (7).

TABLE 1

Desmosine and Isodesmosine Content and
Lysyl Oxidase Acitvity of Control and Blotchy Mouse Lungs

Desmosine and Isodesmosine				Lysyl Oxidase Activity	
Control		Blotchy		Control	Blotchy
n mol/lung	n mol/mg prot.*	n mol/lung	n mol/mg prot.*	cpm/mg prot.	cpm/mg prot.
32.6	1.21	20.4	0.83	296	62
25.3	1.08	18.2	0.63	510	0
28.1	1.14	18.3	0.68	217	0
28.6	1.08	21.0	0.79	247	108

*Based on the saline extracted freeze dried weight of the whole lung.

RESULTS AND DISCUSSION

Desmosine and isodesmosine were decreased in Blotchy mouse lungs as compared to normal (table 1). This relationship was identical whether calculated on the basis per lung or per milligram of lung protein. These results differ from the aortic elastin findings of Rowe et al (1) who reported increased desmosines in Blotchy elastin. The decrease in desmosines was not a result of small lung size. The average body weight of the Blotchy mice was slightly less (23.1 gm) than the controls (25.7), but the average dry weight of the lungs was the same (26.6 gm and 25.4 gm).

Table 1 shows the results of lysyl oxidase assays from 4 Blotchy and 4 normal mouse lungs. A marked decrease in lysyl oxidase activity was observed in extracts from the Blotchy lungs. Only two of the Blotchy lung extracts contained measurable enzyme activity. It is not certain, however, that this result actually proves the absence of lysyl oxidase in Blotchy lungs. Soluble collagen and elastin are readily extracted together with the enzyme during the extraction in 4 M urea. This was illustrated by the method used to prepare the substrate in which the newly synthesized tritium labeled substrate,

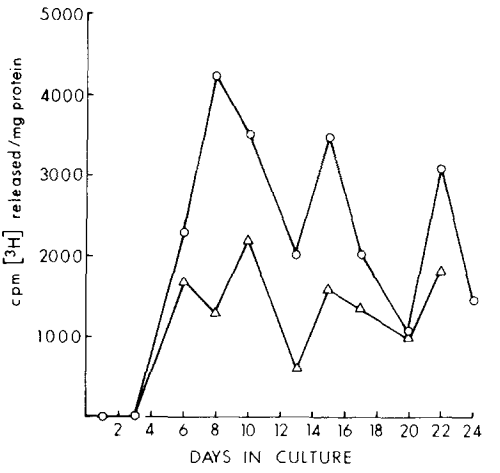


Figure 1

Lysyl oxidase production from normal (O) and Blotchy (△) untransformed (second pass) fibroblast.

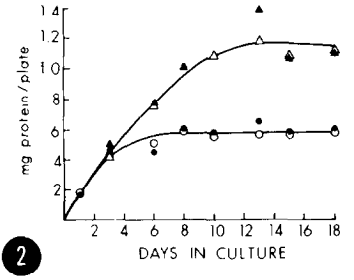
presumably both collagen and elastin, were extracted with 4 M urea. Since the Blotchy mice have increased soluble collagen and uncrosslinked elastin, significant amounts of these proteins may be extracted with the 4 M urea buffer and compete with the labeled substrate. No significant lysyl oxidase activity could be detected in the phosphate buffered saline extract.

Similar difficulties were encountered by Rowe et al (4) when measuring lysyl oxidase in homogenates of mouse skin. They were able to separate inhibitors from the lysyl oxidase prior to assay by affinity chromatography.

Approaching the problem another way, we measured lysyl oxidase activities from fibroblasts in cell culture. Previous studies had shown that lysyl oxidase was secreted by human skin fibroblasts in culture (8) and participated in cross-link formation of collagen secreted by the cells (9). A representative experiment showing lysyl oxidase production from normal and Blotchy mouse fibroblasts on second pass is illustrated in figure 1. Lysyl oxidase in the media from the Blotchy fibroblasts was present at a level only 42% as much as found with fibroblasts from the normal mice. Assay results of fibroblast cultures from three separate Blotchy mice consistently

yielded less than half as much lysyl oxidase as the controls. Lysyl oxidase activity following addition of equal amounts of media from Blotchy fibroblasts, to media from normal fibroblasts was additive, suggesting that the lowered activity seen from the Blotchy cells was not due to the presence of an inhibitor. It was necessary to measure enzyme activity from the initiation of culture until the cells became confluent. Peak activity of lysyl oxidase always occurred just prior to confluence of the cells. Following the time course of the production of lysyl oxidase during the entire period of culture was important because variations occurred between different cultures in the times required for the cells to become confluent. These variations may have resulted from differences between animals or minor differences in the conditions of culture.

In the first subculture following primary culture, the Blotchy cells appeared quite different from the normals. They were less well ordered and exhibited large empty areas between the cells. After one month, however, the Blotchy cells grew as well as the control cells and one could not distinguish between them visually. Whether this early difference in morphology related to lysyl oxidase deficiency or collagen metabolism was uncertain. It was possible that the decreased lysyl oxidase activity of the Blotchy mouse fibroblasts might simply reflect abnormal growth and development of these cells in culture. It is known, however, that mouse fibroblasts undergo spontaneous transformations after months in culture. With such transformations the cells multiply more rapidly and eventually grow at a sustained rapid rate. We have maintained Blotchy and control fibroblasts in culture for several months. After three months, we observed such a transformation in both cell lines. The change was more dramatic with the Blotchy line than with the normals since the Blotchy fibroblasts were so visibly different in the early cultures. Figure 2 shows the marked change in cellular protein between one month old (second pass) cell cultures and cells that had been maintained for four months in culture and had undergone six passes. Interest-



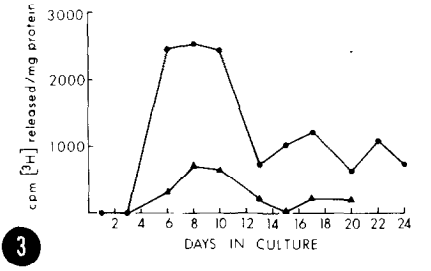


Figure 2

Comparison of total cellular protein between young (untransformed) and transformed fibroblast in culture. (○) Normal untransformed cells. (●) Blotchy untransformed cells. (△) Normal transformed cells. (▲) Blotchy transformed cells.

Figure 3

Lysyl oxidase production from transformed normal (●) and Blotchy (▲) fibroblast.

ingly, the lysyl oxidase levels of the transformed Blotchy cells remained at less than half of the values found in the normal fibroblasts (figure 3). This seems to indicate that the deficiency of lysyl oxidase was inherent for the Blotchy fibroblasts since it persisted after many passes and continued to be expressed even after the cells had undergone transformation. We believe our findings support the idea of a genetically transmitted deficiency of lysyl oxidase in the Blotchy mouse. Presumably, this is the metabolic defect that leads to decreased cross-link formation of elastin and to the appearance of pulmonary emphysema in these animals.

ACKNOWLEDGMENT

This work was supported by a grant from the National Heart and Lung Institute, #HL 16118-04. The authors gratefully acknowledge Dr. W. S. Sly for his assistance in the initial studies and his helpful discussions on the techniques of fibroblast cell culture.

REFERENCES

1. Rowe, D. W., McGoodwin, E. B., Martin, G. R., Sussman, M. D., Grahn, D., Faris, B. and Franzblau, G. (1974) J. Expl. Med. 139: 180-192.
2. Searle, A. G. (1968) Logos International, Plainfield, NJ.
3. Fisk, D. E. and Kuhn, C. (1976) Am. Rev. Resp. Dis. 113: 787-797.

4. Rowe, D. W., McGoodwin, E. B., Martin, G. R. and Grahn, D. (1977) J. Biol. Chem. 252: 939-942.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 139: 265-269.
6. Pinnell, S. R. and Martin, G. R. (1968) Proc. Natl. Acad. Sci. 61: 708-714.
7. Starcher, B. C. (1977) Anal. Biochem., in press.
8. Layman, D. L., Narayanan, A. S. and Martin, G. R. (1972) Arch. Biochem. Biophys. 149: 97-101.
9. Layman, D. L., McGoodwin, E. B. and Martin, G. R. (1971) Proc. Natl. Acad. Sci. 68: 454-458.