Biochimica et Biophysica Acta 891 (1987) 145–149
Elsevier

BBA 42511

Cytochrome a_3 deficiency in human achondroplasia

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(Received 14 October 1986)

Key words: Cytochrome a_3 deficiency; Achondroplasia

Mitochondria prepared from tissue culture cells (skin fibroblasts) from normal subjects and subjects with homozygous achondroplasia were studied to determine the concentrations of cytochromes *a* and a_3 in the preparations. Cytochrome a_3 was markedly decreased (80%) in the achondroplastic preparations with cytochrome *a* present in normal amounts. Determination of total heme *a* (as the pyridine hemochromogen) in the normal and achondroplastic preparations demonstrated that the observed decrease in concentration of cytochrome a_3 in the achondroplastic preparations was due to an absence of cytochrome a_3 and not to a change in its absorbancy (extinction coefficient). The decreased concentrations of cytochrome a_3 in the achondroplastic cells may decrease the reactivity or affinity of the mitochondrial oxidative systems for oxygen and result in the phenotypic expression of the disease.

Introduction

Human achondroplasia is a condition caused by a mutant gene inherited as an autosomal dominant (Ac/ac). The homozygous state (ac/ac) is rare and usually lethal within a few months after birth. Recently, we have shown that concentrations of cytochromes $a + a_3$ were significantly lower in tissue culture cells (skin fibroblasts) from subjects with homozygous achondroplasia than in cells from normal subjects (Mackler, B., Grace, R., Davis, K.A., Shepard, T.H. and Hall, J.G. (1986) Teratology 33, 9–13), although levels of cytochrome *b*, cytochromes $c + c_1$ and cytochrome *c* oxidase activity were normal in the achondroplastic cells. Levels of cytochromes $a + a_3$ in heterozygous achondroplastic cells were intermediate between concentrations in normal cells and homozygous achondroplastic cells as would be expected from the effects of gene dosage. The present work was undertaken to determine whether cytochrome *a* or a_3 or both were decreased in the homozygous achondroplastic cells.

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Materials and Methods

The human skin fibroblast strains from normal subjects and from subjects with homozygous achondroplasia used in the studies were those described previously by Mackler et al. [1]. The infants with homozygous achondroplasia from whom skin biopsies were obtained were born to parents who both had heterozygous achondroplasia. The infants had extremely small chests and the diagnosis of homozygous achondroplasia was confirmed in both cases by the X-ray examination which showed the classical findings of homozygous achondroplasia with flattening of the vertebrae, severe contraction of the base of the skull, markedly short long bones, severely contracted pelvis, and a relatively large cranium.

Tissue culture methods were as previously described by Hakami and Pious [2] and Pious [3]. The culture medium was Medium 199 (Gibco Laboratories, Santa Clara, CA) supplemented with 15% fetal bovine serum. Experiments with the various strains of cells were performed with cell cultures which had undergone approximately the same number of doublings (14–20 doublings) in order to minimize any possible effects of aging of individual cell lines on the experimental results.

Mitochondria were prepared from the various strains of fibroblasts cultured aerobically in small roller bottles (690 cm^2 surface area per bottle; eight roller bottle cultures per experiment) as described by Kobayashi et al. [4]. Fibroblast submitochondrial particles were prepared by sonicating mitochondria suspended in 0.25 M sucrose (3×20 s at 120 W with a Braunsonic 1510, B. Braun Instruments, South San Francisco, CA 94080, using a 'needle probe'). After centrifugation at $2000 \times g$ for 10 min, the submitochondrial particles were precipitated by centrifugation at $106\,000 \times g$ for 30 min. The particles were suspended in 20 mM phosphate (K) buffer (pH 7.5) containing 0.12% Tween 20. Bovine heart mitochondria were prepared by the method of Blair [5].

The absorption of cytochromes $a + a_3$ at 605 nm (605 minus 620 nm) in the mitochondrial and submitochondrial particulate preparations was calculated from difference spectra (dithionite reduced minus oxidized) determined with an aminco DW-2 recording spectrophotometer. The absorbance contribution of cytochrome a at 605 nm (605 minus 620 nm) was then determined from CO reduced (dithionite) minus oxidized difference spectra on the same mitochondrial preparations by bubbling CO slowly through the reduced sample for 1 min and then recording the difference spectra. The absorbance contribution of cytochrome a_3 at 605 nm was calculated for each preparation as the difference between the absorbance value for cytochromes $a + a_3$ (determined from the reduced minus oxidized difference spectrum) and the absorbance value for cytochrome a (determined from the CO reduced minus oxidized difference spectrum).

Concentrations of cytochrome b were calculated from the difference spectra (dithionite reduced minus oxidized; 562–570 nm) using the millimolar extinction coefficient of 20 [6].

Total heme a was determined from difference spectra (dithionite reduced minus peroxide oxidized) at 587–620 nm of the pyridine hemochromogens prepared from preparations of submitochondrial particles by the method of Morrison and Horie [7].

Succinic dehydrogenase activity was determined spectrophotometrically at 25°C as previously described [8]. Protein concentrations were determined by the method of Lowry et al. [9]. Statistical analyses were performed by standard methods of variance as described by Snedecor and Cochran [10]. All chemicals were obtained from the Sigma Chemical Co., St. Louis, MO.

Results and Discussion

Previously, Vannesta has shown that cytochrome a_3 concentrations may be determined separately from cytochrome a levels in preparations of bovine heart cytochrome c oxidase and Keilin–Hartree preparations by means of CO reduced minus reduced difference spectra recorded in the Soret region of the spectrum [11]. Fig. 1 shows CO reduced minus reduced (dithionite) difference spectra of preparations recorded as described by Vannesta of bovine heart mitochondria and mitochondrial preparations from normal and achondroplastic tissue culture cells. As shown in the figure the spectrum of bovine heart preparation has a peak indicative of cytochrome a_3 with a maximum at 427 nm and a minimum at 445 nm in accord with the findings of Vanneste. However, preparations of mitochondria from both normal and achondroplastic cells have in addition a large peak at 416 nm which obscures the 427 nm peak of cytochrome a_3 and interferes with the determination of the cytochrome concentrations by this method. It is of interest, however, that the 427 nm shoulder (indicative of cytochrome a_3) of the 416 nm peak appears much larger in the spectrum of the normal mitochondria than in the spectrum of the achondroplastic mitochondria suggesting that the cytochrome a_3 concentration is lower in the achondroplastic preparation although an accurate determination cannot be made.

Since spectra recorded in the Soret region were unsatisfactory for determination of concentrations

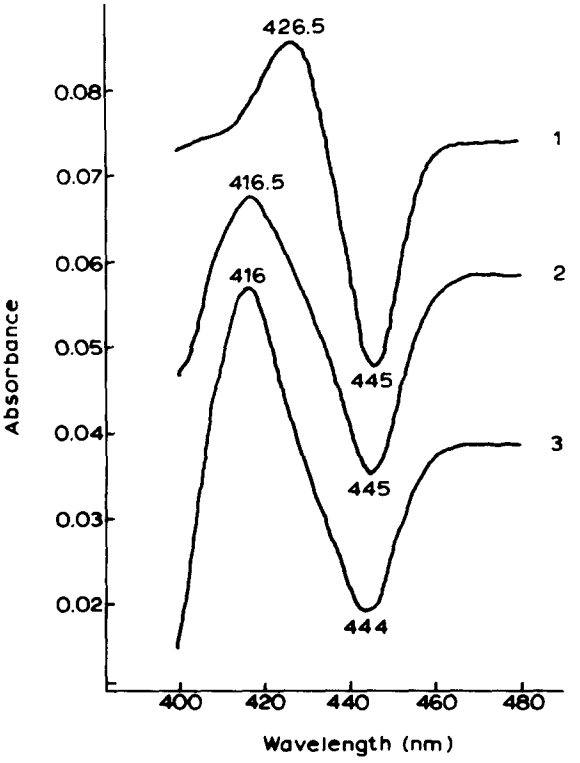

Fig. 1. CO-reduced (dithionite) minus reduced difference spectra for mitochondrial preparations from (1) bovine heart (2.5 mg protein per ml), (2) normal tissue culture cells (2.3 mg protein per ml) and (3) achondroplastic tissue culture cells (2.1 mg protein per ml). All preparations were suspended in a 0.05 M solution of Tris-HCl (pH 8.0).

of cytochrome a_3 and a in mitochondrial preparations from normal and achondroplastic tissue culture cells, it was necessary to determine the cytochrome a and a_3 concentrations from difference spectra at 605 nm as described in Methods and the results are shown in Table I. Representative spectra from an experiment on normal mitochondria and an experiment on achondroplastic mitochondria are shown in Figs. 2 and 3, respectively. Concentrations of cytochrome b and succinic dehydrogenase activity were determined in the preparations as a measure of the relative purity of the preparations and as shown in Table I no significant difference ($P > 0.2$) was found between the normal and achondroplastic preparations. In accord with our previous findings [1] levels of cytochromes $a + a_3$ were significantly lower ($P < 0.05$) in preparations from both achondroplastic cell lines than in the normal preparations. The further finding (as shown in Table I) that levels of cytochrome a (determined from CO-treated reduced minus oxidized difference spectra) were not significantly different ($P > 0.4$) in the normal and achondroplastic mitochondrial preparations demonstrated that the lower levels of cytochromes $a + a_3$ in the achondroplastic preparations were due to a marked decrease (approx. 80%) in the levels of cytochrome a_3. It is of interest that in accord with previously reported work [11–14] the contribution of cytochrome a_3 to the absorbtion peak at 605 nm is less than 50% in the normal preparations. However, in the above experiments the contribution of cytochrome a_3 is significantly greater (approx. 40%) than that reported by the other workers (approx. 20%) and this difference may be explained by tissue variations in the composition and activity of the

TABLE I

ABSORBTION OF CYTOCHROMES a AND a_3 AT 605 nm IN MITOCHONDRIAL PREPARATIONS FROM NORMAL AND HOMOZYGOUS ACHONDROPLASTIC TISSUE CULTURE CELLS

Cell type	Succinic dehydrogenase activity *	Cytochrome b * (pmol per mg protein)	Cytochromes * a and a_3 (absorbtion $\times 10^3$ per mg protein)			a_3
			$a + a_3$	a	a_3	$a + a_3$
Normal 1	11.4 ± 3.4	131 ± 15	1.68 ± 0.02	1.02 ± 0.11	0.66 ± 0.10	0.39 ± 0.2
Normal 2	16.7 ± 1.1	123 ± 12	1.53 ± 0.04	0.97 ± 0.04	0.56 ± 0.08	0.37 ± 0.04
ac/ac 1	13.2 ± 0.7	122 ± 6	1.04 ± 0.09	0.93 ± 0.06	0.11 ± 0.03	0.11 ± 0.02
ac/ac 2	13.7 ± 1.9	110 ± 8	1.19 ± 0.11	1.02 ± 0.09	0.17 ± 0.05	0.14 ± 0.4

* The values are expressed as the mean of three separate experiments for each normal cell type and four separate experiments for each achondroplastic cell type ± the standard error of the mean. Succinic dehydrogenase activity is expressed as nmol succinate oxidized per min per mg protein.

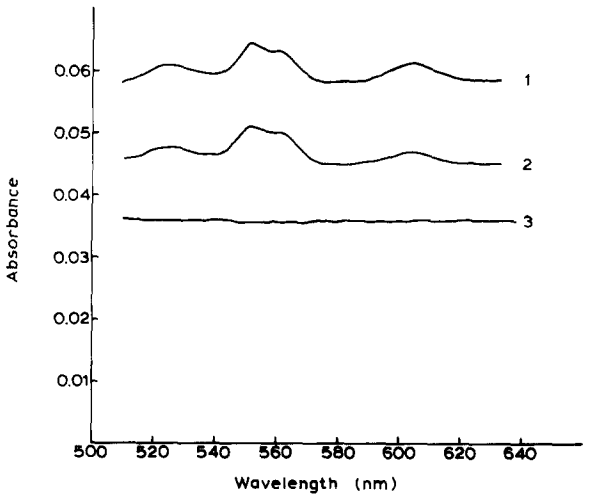

Fig. 2. Difference spectra determined for a representative experiment on mitochondria from normal tissue culture cells. (1) Reduced (dithionite) minus oxidized spectrum. (2) Reduced + CO minus oxidized spectrum. (3) Baseline. The curvettes contained 2.18 mg of mitochondrial protein in 1 ml 50 mM potassium phosphate buffer (pH 8.0).

mitochondrial electron transport systems, since other investigators studied only preparations from heart muscle. Since previously determined extinction coefficients for cytochromes a and a_3 were calculated from data obtained from heart muscle preparations and assumed a 20% contribution of cytochrome a_3 to the total absorbance at 605 nm [15,16], we were unable to use them for calculation of molar concentrations of cytochromes a and a_3 in the tissue culture cell preparations, and have, therefore, expressed the cytochrome concentrations as absorbancy per mg of mitochondrial protein. The decrease in concentrations of cytochrome a_3 observed above in achondroplastic preparations may be due either to an actual decrease in the amount of cytochrome a_3 present in the preparations or to a change in the absorbancy (extinction coefficient) of the cytochrome due to abnormal binding to an altered cytochrome oxidase. To study this further, concentrations of cytochrome $a + a_3$ and concentrations of total heme a were determined sequentially on the same samples of normal and achondroplastic preparations of submitochondrial particles and the results are expressed as the ratio of the absorbance of the heme a (587–620 nm) divided by the absorbance of cytochrome $a + a_3$ (605–620 nm) for each preparation. Ratios of 1.6 and 1.7 were determined for the two control fibroblast lines whereas a ratio of 1.4 was determined for each of the achondroplastic cell lines. These findings are in agreement with a decreased heme a content in the achondroplastic lines rather than a cytochrome a_3 with altered spectral characteristics. This is evident, since using the ratio of heme a absorbance to cytochrome $a + a_3$ absorbance of 1.6 for the normal cell lines as determined above and a 40% contribution of cytochrome a_3 to the absorption of cytochromes $a + a_3$ at 605 nm as shown in Table I, a theoretical ratio of 1.4 was calculated assuming cytochrome a_3 was decreased 80% (see Table I) in the achondroplastic preparations. This ratio is in accord with the ratio of 1.4 determined experimentally for the achondroplastic preparations. Alternatively, a theoretical ratio of 2.4 was calculated assuming that the observed decrease in cytochrome a_3 was due to normal levels of the cytochrome but altered optical properties.

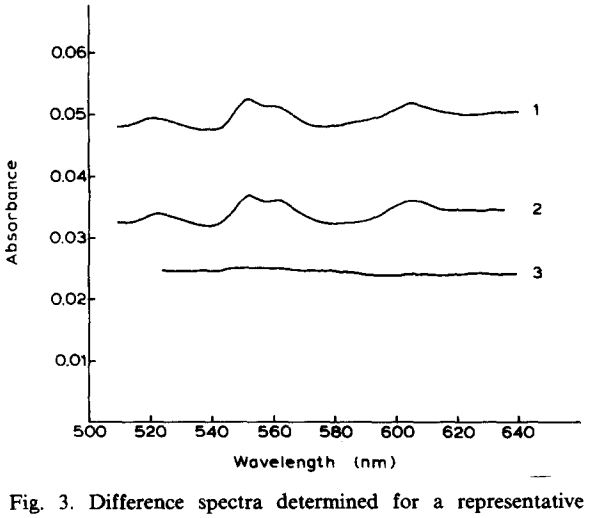

Fig. 3. Difference spectra determined for a representative experiment on mitochondria from achondroplastic tissue culture cells. (1) Reduced (dithionite) minus oxidized spectrum. (2) Reduced + CO minus oxidized spectrum (3) Baseline. The cuvettes contained 1.76 mg of mitochondrial protein in 1 ml 50 mM potassium phosphate buffer (pH 8.0).

Achondroplasia is inherited as a dominant autosomal trait and is manifested primarily by defective endochondrial bone growth. Resting cartilage is responsible for growth of the long bones and has the lowest oxygen concentrations of the body

tissues [17]. It is, therefore, possible that the mutant gene (ac) may be expressed as an inability of achondroplastic cartilage cells to grow normally at very low oxygen concentrations due to the markedly reduced concentrations of cytochrome a_3 which result in decreased reactivity or affinity of the mitochondrial oxidative systems for oxygen with decreased energy production and growth of the cells.

Acknowledgements

The work was supported in part by grants GM-23006 and HD-02274 from the National Institutes of Health.

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