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COMPARATIVE STUDY OF CYTOCHROMES BETWEEN VIRUS-TRANSFORMED AND UNTRANSFORMED CELLS

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SUMMARY

Tissue culture cells of virus-transformed and untransformed cell lines had low contents of cytochromes in the respiratory chain, measured per cell or per mg protein of the cells, in comparison to the cytochrome contents of liver cells *in vivo*.

In the virus-transformed cells the contents of cytochromes aa_3, b and possibly c_1 were significantly lower than those in the untransformed cells, while the content of cytochrome c was found to be the same or even increased in the transformed cells. Thus, a markedly high ratio of cytochromes c/aa_3 was observed in the transformed cells.

Polarographic measurements of the oxygen uptake have shown a generally low rate of both endogenous respiration and respiration in the presence of glucose and vitamin K_3 in the transformed cells.

The present study indicates that there is a quantitative and possibly qualitative alteration of the respiratory chain components in the transformed cells.

INTRODUCTION

Since Warburg demonstrated that a low rate of respiration accompanied a high glycolytic capacity in tumor cells[1,2], attempts have been made to elucidate the relationship between these two phenomena[3–6]. The general conclusion of Chance and Hess[6] was that tumor cells differed in control mechanism rather than in contents of cytochromes or their turnover numbers. However, the low rate of respiration has been explained, at least partially, by the low mitochondrial content of tumor cells[7,8]. There is a lower cytochrome content in the mitochondria of hepatoma cells (with the exception of cytochrome c) than in non-malignant liver cells[9], which

Abbreviation: SV 40, Simian virus 40.

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suggests that there may be an alteration of mitochondrial membrane structure and/or heme synthesis in these cells. These findings, however, are limited in their application because the interference of normal or unidentified cells may affect the determination of the content of cytochromes in tumor cells. For an accurate comparison of the cytochrome spectra in tumor cells with the cytochrome spectra in normal cells, therefore, it would appear advantageous to examine the same cell line before and after malignant transformation.

This communication details a spectrophotometric analysis of the cytochrome contents of several tissue culture cell lines transformed by a DNA tumor virus as compared with the untransformed cell lines. The transformation of cells by SV 40 virus causes a significant reduction in the contents of cytochromes aa_3, b and possibly c_1, while the content of cytochrome c remains unaffected or even slightly increases. The data on oxygen uptake, measured polarographically, are also presented. These results suggest the possible impairment of mitochondrial membrane structure and heme synthesis in tumor cells.

MATERIALS AND METHODS

Cell lines

For the experiments, three untransformed and five Simian virus 40 (SV 40)-transformed cell lines of three different species were used. 3T3, Swiss mouse cell line originally obtained from Dr H. Green, Mass. Inst. Tech., Cambridge, Mass. SV-3T3, SV-3T3 is an SV 40-transformed cell culture (Swiss mouse), obtained as clone 101 from Dr H. Green, Mass. Inst. Tech., Cambridge, Mass. MKS-Bu 100, SV 40-transformed kidney cell line Balb-C, resistant to 30 μg/ml 5-bromodeoxyuridine. This was received originally from Dr Del Dubbs, Baylor College of Medicine, Houston, Texas. WI-38; cell line of human embryo lung fibroblasts established by Hayflick in 1965. W 98 Vac; cell line derived by transformation of human skin with SV 40 in 1963. W18Va2; SV 40-transformed culture derived from human buccal mucosa and kept in continuous passage since 1962. CV-1; CV-1 cells are a continuous line of untransformed AGMK cells. AGMK, clone A-10-2; A clone of an γ-irradiated adeno virus 7 SV 40-transformed African Green Monkey kidney cell line. AGMK-VaE, clone 2A-1; a clone of AGMK cells transformed by SV 40. It does not yield virus when fused with AGMK cells (negative clone).

All cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS, Flow Laboratories, Rockville, Md.) and 100 μg/ml of streptomycin and 100 units/ml of penicillin.

The cells were incubated at 37 °C in blake bottles and sometimes in roller bottles. The cells were harvested before the density in the culture reached saturation in the bottles. The cell counts of the untransformed and the transformed cells when harvested were $1 \cdot 10^7$ to $2 \cdot 10^7$ and $3 \cdot 10^7$ to $4 \cdot 10^7$ per bottle, respectively.

Determination of cytochromes

To determine the cytochrome content, a comparison of the difference absorption spectra of the fully oxidized state and the fully reduced state of cytochromes at the temperature of liquid nitrogen was made with a split-beam spectrophotometer[10,11]. For one experiment, approx. $1 \cdot 10^8$ cells suspended in phosphate-buffered saline

were used. Cytochromes were oxidized by adding 5 μM rotenone under aerobic conditions[6] to the reference cuvette. After 1 min, the reference sample was frozen by the trapped steady-state method[12]. A reduction of cytochromes in the assay cuvette was obtained by adding either sodium dithionite to the cells, or by adding KCN in the presence of endogenous substrate to determine the cytochrome contents in the mitochondrial respiratory chain sensitive to cyanide. The absorbance changes were measured at the following wavelength pairs: for cytochrome aa_3, 600 and 630 nm; for cytochrome b, 558 and 575 nm; for cytochrome b_{557}, which is not reduced by addition of cyanide but is reduced by sodium dithionite, 557 and 570 nm; for cytochrome c, 548 and 540 nm. The extinction coefficients used have been given previously[9]. For the determination of cytochrome b_{557}, a value of 17.9 mM^{-1} (ref. 17) was tentatively used. The following intensification factors at low temperature under the present experimental condition were used: for cytochromes aa_3, b and b_{557}, 7.0; for cytochrome c, 8.0. The O_2 uptake of transformed and untransformed cells was measured polarographically with a Clark oxygen electrode.

Protein was determined by the biuret method with bovine serum albumin as the standard[13]. The number of cells was counted by a Burker–Turk hemocytometer.

RESULTS

The spectra of cytochromes of the SV 40-transformed and untransformed cells

The spectra on the right-hand side of Fig. 1 show the difference absorption spectra (reduced − oxidized) of the SV 40-transformed and untransformed mammalian cells taken at the temperature of liquid nitrogen. The reduction and oxidation of cytochromes were obtained by the addition of sodium dithionite and rotenone, respectively. The spectra of the cells show well-defined absorption maxima at 599, 560 and 547 nm, corresponding to the alpha bands of cytochromes aa_3, b and c, respectively. In the spectra of the virus-transformed cells, SV-3T3, W98VaC and 2A-1, the diminished alpha band of cytochrome aa_3 and the increased alpha band of the b cytochromes can be compared with those of the untransformed cells, 3T3, WI38 and CV-1. On the other hand, the absorbance of cytochrome c appears to be similar or somewhat increased in the transformed cell lines. From these spectra, the contents of cytochromes aa_3 and c in the transformed and the untransformed cells were calculated and presented in Table I.

When the difference spectrum was taken at 77 °K between KCN-treated cells and rotenone-treated cells (left-hand side of Fig. 1), the absorbances of alpha bands of cytochromes aa_3 and c were almost the same as those in the spectrum obtained by treatment with sodium dithionite, except for the appearance of a cyanide–aa_3 complex at 587 nm in the KCN-treated cells. However, the absorbance around 555–560 nm is greatly diminished in the spectra of KCN-treated cells as compared to those of dithionite-treated cells. Since the addition of cyanide to the respiring yeast cells causes full reduction of cytochrome b_K (alpha band at 558 nm at 77 °K, ref. 14) and partial reduction of cytochrome b_T (alpha band at 562 nm at 77 °K, refs 14, 15), the content of cytochrome $b_{(K)}$ in the respiratory chain was calculated from the difference in absorbance between 558 nm and 575 nm in the spectra of KCN-treated cells. In transformed cells, a reduction of the cytochrome $b_{(K)}$ band is generally seen as compared with the untransformed cells (left side of Fig. 1 and Table I).

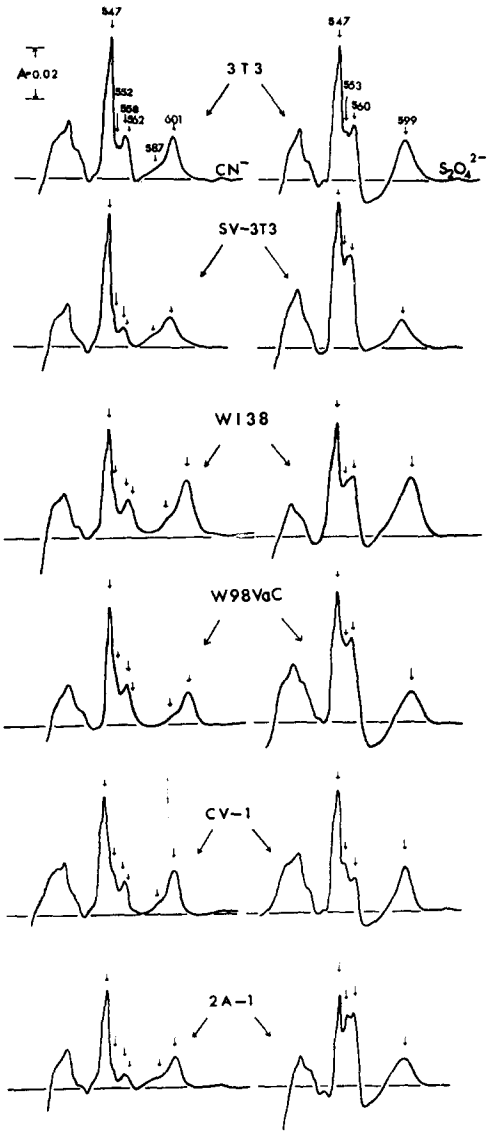

Fig. 1. The oxidized–reduced absorption spectra of cytochromes from SV 40-transformed and untransformed cell lines. The 3T3, SV-3T3, WI38, W98VaC, CV-1 and 2A-1 cells were suspended in phosphate-buffered saline at 40, 40, 30, 31, 36 and 35 mg protein/ml, respectively. Each reference sample was supplemented with 5 μM rotenone to oxidize the cytochromes in the respiratory chain, withdrawn 1 min later, and injected into a spectrophotometer cuvette which had been precooled to the temperature of liquid nitrogen[12]. Each measuring sample of the spectrum on the right-hand side of the figure had been treated with sodium dithionite, withdrawn 2 min later and treated in the same way as the reference sample. The measuring sample of the spectrum on the left-hand side of the figure was treated with 2 mM KCN, shaken in the air, withdrawn and injected into the cuvette. Light path, 0.2 cm; temperature, 77 °K.

TABLE I

CYTOCHROME CONTENTS OF THE TRANSFORMED AND UNTRANSFORMED CELLS CULTURED *IN VITRO*

The contents of cytochromes were calculated from the reduced – oxidized difference spectra at 77 °K. The content of cytochrome b_{557} was calculated from the difference spectrum of dithionite-treated *minus* KCN-treated cells at 77 °K. The numbers in brackets indicate the number of experiments.

Cells	*Characteristics*	*Content (nmoles/g protein)*				*g protein (10^9 cells)*
		Cyto-chrome aa_3	*Cyto-chrome $b_{(K)}$*	*Cyto-chrome c*	*Cyto-chrome b_{557}*	
3T3 (3)	untransformed	20±3*	14±4	46±8	14±4	3.3
SV-3T3 (3)	transformed	15±2	9±4	52±8	28±6	3.4
WI38 (3)	untransformed	27±5	17±3	40±4	16±6	3.8
W98VaC (3)	transformed	15±2	13±2	50±6	19±3	3.8
W18Va2 (1)	transformed	14	10	49	—	3.9
CV-1 (3)	untransformed	23±4	12±2	51±9	6±1	3.0
A-10-2 (1)	transformed	17	12**	49	40	—
2A-1 (3)	transformed	19±6	10±2	52±6	33±4	3.0
Liver***	normal rat	122±9	78±8	103±12	—	—

* Mean value ±S.E.
** A partial contribution of cytochrome c_1 is included in this value.
*** Ref. 9.

Cytochrome c_1 is seen at 552 nm in the spectra of cyanide-treated cells. This cytochrome seems to decrease parallel to the decrease of cytochrome $b_{(K)}$ in the transformed cells, although this cannot be positively determined due to the small absorbance change of cytochrome c_1 and the interference of the high absorbance change of cytochrome *c*.

When the difference spectrum between dithionite-treated cells and cyanide-treated cells was taken at 77 °K, the alpha absorption band is seen at 557 nm with shoulders at 561 and 551 nm (Fig. 2). The beta and Soret bands lie at 528 nm and 424 nm, respectively. A part of cytochromes b_T and c_1 may be involved in these spectra. The 557-nm-absorbing pigment will be designated as cytochrome b_{557} (*cf.* ref. 16). The content of cytochrome b_{557} is calculated from the difference in absorbance at 557-nm *minus* 570 nm (Table I).

Comparison of cytochrome contents between transformed and untransformed cells

The cytochrome content, expressed per mg of protein or per cell, is generally low in the respiratory chain of tissue culture cells (Table I), when compared with that of normal rat liver cells *in vivo*[9,18,19]. The value is somewhat comparable to that of ascites tumor cells[9]. The ratio of cytochrome *c* to cytochrome aa_3 is higher (1.5–3.6) in all tissue culture cells than that (0.8–1.8*) in non-malignant cells *in vivo*[9,19,20].

When the cytochrome content in the respiratory chain of the transformed and the untransformed cell lines are compared, the contents of cytochromes aa_3

* 1.8 represents the combination of cytochromes $c+c_1$.

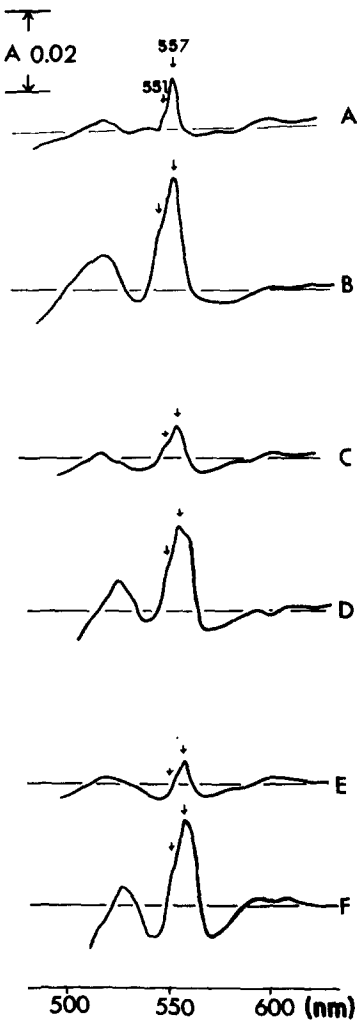

Fig. 2. The spectra representing the difference in absorption between sodium dithionite-treated cells and KCN-treated cells. The 3T3 (A), SV-3T3 (B), WI38 (C), W98VaC (D), CV-1 (E) and 2A-1 (F) cells were suspended in the same way as in Fig. 1. Each reference sample was treated with 2 mM KCN and treated as in the legend of Fig. 1. Each measuring sample was treated with sodium dithionite and treated as in the legend of Fig. 1. Therefore, the measuring sample corresponds to the measuring one on the right-hand side of Fig. 1, and the reference sample corresponds to the measuring sample of the respective left-hand side of Fig. 1. Light path, 0.2 cm; temperature, 77 °K.

and $b_{(K)}$ are, in general, low in the transformed cell lines. The content of cytochrome *c* is, however, almost the same or somewhat higher than that of the untransformed cells. When the ratios of cytochrome $b_{(K)}$ or *c* to aa_3 are calculated, b/aa_3 is roughly equal in both the transformed and untransformed cells. The ratio c/aa_3, however,

is remarkably increased in the transformed cells (2.6–3.6), a finding consistent with that obtained in ascites cells[9].

The cytochrome(s) which is not reduced by the addition of KCN to the cell suspension but which is reducible by dithionite (cytochrome b_{557}) is, in all transformed cells tested, increased in amount(s). In the monkey kidney transformed cell lines, 2A-1 and A-10-2, it is 6–7 times as high as that of the untransformed CV-1 cells. The significance of the increased amount of cytochrome b_{557} is not known.

The respiratory rate of tissue culture cell lines

Polarographic measurements of the oxygen uptake of transformed and untransformed cells were made to determine a possible correlation between the diminution of cytochromes aa_3, $b_{(K)}$ and c_1 and respiratory impairment in these tissue culture cells. The rate of endogenous respiration and the respiratory rate in the presence of glucose and vitamin K_3 are presented in Table II. These tissue culture

TABLE II

THE RESPIRATORY RATE OF TISSUE CULTURE CELLS

The tissue culture cells were suspended in phosphate-buffered saline equilibrated in air at 25 °C. The respiratory rate of the cells was calculated in the absence and in the presence of glucose and vitamin K_3 using an O_2 electrode. The additions were 2 mM glucose and 25 μM vitamin K_3. n.c., not calculated.

Cells	*Respiration (nmoles O_2/min per 10^8 cells)*	
	Endogenous	*Glucose vitamin K_3*
3T3	70	170
SV-3T3	30	100
MKS-Bu100	55	90
WI38	n.c.	710
W98VaC	n.c.	140
HeLa	180	260

cells have a generally low rate of endogenous respiration, especially in the case of the virus-transformed cells. Glucose and vitamin K_3 activate the respiration of both the transformed and the untransformed cells. In this case the electron may pass from substrates *via* DT diaphorase in the presence of vitamin K_3 (ref. 21) to the respiratory chain. The rate of respiration is, however, still lower, though not so significantly, in the presence of glucose and vitamin K_3 in the transformed cells than in the untransformed cells. It seems likely that the lowered concentrations of cytochromes in the transformed cells resulted in the decreased rate of respiration.

DISCUSSION

The present spectrophotometric study has revealed low contents of cytochromes in the respiratory chain per cell or per mg protein in the tissue culture cell lines in comparison to the cytochrome content of normal liver cells *in vivo*[9,18]. The amounts of cytochromes found in this study are comparable to the reported

values for tissue culture L cells[22] and for fibroblast[27]. The finding that the explantation of tissue into culture causes a loss or decrease of total enzyme activity or content[23] is compatible with the data on the low cytochrome content in cultured cells.

It seems more interesting to find that the lowering of the contents of cytochromes, especially cytochrome aa_3, is greater in the transformed cells than in the untransformed cells. The observations that ascites tumor cells have a low cytochrome aa_3 content and a high c/aa_3 ratio[9] and that carcinogenesis in rat liver by feeding *p*-dimethylaminoazobenzene is accompanied by a gradual decrease of cytochrome aa_3 content and the gradual increase in the ratio of c/aa_3 (Oyanagui, Y., Sato, N. and Hagihara, B., unpublished results) are also consistent with the present finding in the virus-transformed cells. Therefore it could perhaps be said that tumors differ from normal cells in the content of cytochromes in the respiratory chain as well as in the control mechanism[6]. The decreased content of cytochromes could result in the decrease in the respiratory rate in the tumors *in vivo*.

The cytochrome content in cells seems to depend on the cellular environment[24–26] and their pathological condition[9,31,32]. Since anaerobically grown yeast[24] and human cells[25] have a low cytochrome oxidase activity as compared with those grown aerobically, it is possible to attribute the low cytochrome aa_3 content in tissue culture cells to the oxygen environment in which the cells are grown. The observed low contents of cytochromes aa_3, $b_{(K)}$ and c_1 in the transformed cells might result from the relatively low oxygen tension in the denser transformed cultures. This possibility, however, may be unlikely. The cytochrome contents in the cells cultured in the roller bottle were found to be almost equal to those cultured in the blake bottle; in the roller bottle the cells were almost always exposed to the air during culture, while in the blake bottle the cells were in the culture medium. The c/aa_3 ratio was identical in both cases (Kato, K. and Sato, N., unpublished results). It was reported that a lowered concentration of oxygen in the culture medium resulted in a decrease in the content of all the cytochromes (aa_3, b and $c+c_1$) in the respiratory chain in mammalian cells[27,28]. Our study revealed that, in the transformed cells, the cytochromes aa_3, $b_{(K)}$ and c_1 levels decreased while the cytochrome c level was unaltered or increased. However, it is not ruled out that small difference in the culture condition, such as oxygen tension and glucose concentration, may effect the synthesis of each cytochrome component in the respiratory chain with or without enhanced degradation.

The observation of changes in the cytochrome composition of cells has indicated that cytochrome aa_3 or cytochrome oxidase is most easily affected, and that cytochrome c is least affected, by environmental change or by pathological conditions. This may be due to differences in the biosynthetic process of each cytochrome. The quantitative, and possible qualitative, alteration of cytochromes in the mitochondrial respiratory chain may have some relation to the inefficiency of mitochondria[29] resulting in the high rate of aerobic glycolysis in tumors.

The *b* cytochrome with an alpha band at 557 nm was increased in amount by the malignant transformation of the cell. This cytochrome is spectrally different from the cytochrome *b* in the mammalian[14] or yeast[15] mitochondrial respiratory chain and different from the cytochrome b_5 in liver microsomes[30]. This 557-nm-absorbing pigment(s) might be a modified form of cytochrome in the respiratory chain. The appearance of this pigment may be related to a disorganization of the

mitochondria, since, in the cells treated with ethidium bromide, its amount increased significantly at the same time as decreases in the amounts of respiratory cytochromes[16] and the appearance of abnormally organized cristae[33,34]. The presence of similar *b*-type cytochromes has also been reported in ascites tumor cells[9] and in anaerobically grown yeast[35].

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