DECREASED RATE OF S-ADENOSYL-L-HOMOCYSTEINE METABOLISM : AN EARLY EVENT RELATED TO TRANSFORMATION IN CELLS INFECTED WITH ROUS SARCOMA VIRUS

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SUMMARY. An increase of methylase activity is often related to neoplastic transformation. SAH, the natural inhibitor of transmethylases, does not inhibit cell transformation induced by RSV, in contrast to one of its synthetic analogues, SIBA. This inefficiency was thought to be due to the rapid metabolism of SAH by transformed cells. We now show, that, on the contrary, 70 % of the added amount of SAH disappears in one hour in cell-free extracts of normal cells against only 14 % in extracts of transformed cells. This decreased rate of degradation occurred one day post infection. Cells infected with the non transforming RAV_1 degrade SAH at the same rate as normal cells. A decrease of SAH-hydrolase and adenosine deaminase activity was also observed in infected cells. The decrease of the first enzyme seems to be related to the transformed state, whereas that of the second enzyme seems to depend only on infection, since it is also observed in cells infected with RAV_1.

INTRODUCTION

Increased levels of methylase activities in animal and human tumors have been reported (1,2). Methyltransferases may also be implicated in cell transformation induced by oncogenic viruses (3,4).

If one assumes that hypermethylations play a decisive role in cell transformation, specific inhibitors of methyltransferases should interfere with the oncogenic process. However, although S-adenosyl-L-homocysteine (SAH), one of the products of enzymatic transmethylation, is a potent inhibitor of tRNA methyltransferases in vitro (5) its effect on cell transformation is insignificant (6). Furthermore, we showed that S-isobutyl-adenosine (SIBA), a synthetic analogue of SAH, prevents oncogenic transformation of chick embryo fibroblasts (CEF) by Rous Sarcoma Virus (RSV), although it is a weak inhibitor of tRNA methyltransferases in vitro compared to L-SAH (6). In the same publication, we

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Abbreviations :
SAH : S-adenosyl-homocysteine ; SIBA : 5'-S-isobutyl adenosine ; RAV : Rous Sarcoma associated virus ; RSV : Rous Sarcoma virus ; CEF : chick embryo fibroblasts; SAM : S-adenosyl-methionine ; SRC gene : the transforming gene of avian tumor viruses ; Tris : Tris-(hydroxy-methyl)aminomethane.

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reported that SAH and SIBA have the same cytostatic effect on normal cells,which could be reversed by renewal of the medium. Thus, these two compounds have a similar effect on normal cells but different effects on cell transformation by RSV.

This suggests that there may exist a difference in the level of one or several enzymes which degrade or modify SAH in normal and in transformed cells. The aim of this work was to determine the activity of several enzymes responsible for the modification and for degradation of SAH, to study the rate of the degradation of SAH in cell-free extracts of normal, infected and transformed cells and to compare the effect of SAH and SIBA on methyl group incorporation into nucleic acids of cells in culture.

MATERIALS AND METHODS : Cells and viruses : Secondary cultures of CEF were prepared and cultivated as described by Goldé and Vigier (7), and infected either by a clonal isolate SR 4 of Schmidt-Ruppin strain RSV (SR-RSV) type D, or by a thermosensitive ts mutant Bu57 of SR-RSV, type A, isolated by Dr G. Calothy, Institut du Radium, Orsay. The cells infected with this mutant have the transformed morphology at 37° and normal morphology at 41°. CEF were also infected with a non transforming helper virus of RSV, RAV_1(8). Infection was carried out one day after seeding with adequate amount of virus to obtain 80 % transformation in 4-5 days at 37°, or the infection of all the cells in the case of RAV_1. The established normal rabbit cell line 905 was isolated by Drs R. Sheldrick and M. Laithier (Institut de Recherche sur le Cancer, Villejuif, France). The homologous transformed line, A_2 was obtained by the same authors by infection of 905 cells by the ts mutant Fu 19 derived from SR 4 (9). - The normal cell line C 13/15 was a subclonal derivative of the clonal line BHK 21/C13, isolated by Dr L. Montagnier (Pasteur Institute, Paris). The homologous transformed line RS 2/3, a clonal line was obtained by infection of BHK 21/C13 cells with SR-RSV-D (10).

All the cells were cultivated in Ham F10 (11) medium supplemented with glutamin, antibiotics and 5 % calf serum. During methionine incorporation the serum concentration was reduced to 2 %.

Chemicals. All chemicals used were of the highest purity available and came from the following sources ; SIBA and SAH : Sefochem Fine Chemicals, Emek Hayarden Israel, adenosine, salts, TRIS and β-mercaptoethanol : Merck (Germany), DL-homocysteine : Fluka (Switzerland) S-adenosyl-methionine methyl (^{14}C) 50 mCi/mM and methionine methyl (^{3}H) 11 Ci/mM : Commissariat à l'Energie Atomique, Saclay, France, sodium pyruvate, 5,5'-dithiobis(2-nitrobenzoic acid) : Aldrich (Belgium), 3-hydrazinoquinoline : Eastman Kodak (France) ; E. coli B tRNA : Sigma (USA) Adenosine deaminase (calf intestinal) : Boehringer (Germany).

Preparation of cell free extracts and current assays. The preparation of cell-free extract, the determination of the activity of tRNA methylases and the determination of protein concentration were performed as described previously (6), SAH and SIBA were dissolved in the medium and sterilized by filtration.

(^{3}H) methyl group incorporation. To measure the extent of methylation, exponentially growing normal or transformed cells were labelled during 5 hours at 37° with (^{3}H-CH_3) methionine 11 Ci/mM, 30 µCi/ml in the presence of unlabelled sodium formiate 20 mM, adenosine 20 µM, and guanosine 20 µM to inhibit methyl incorporation into purine and thymine via "one carbon" pool. The supernatants were discarded after labelling and the monolayers washed three times with ice cold buffered saline of the following composition:in 1 litre of bidistilled wa-

TABLE I

Extent of incorporation of (^{3}H)methyl from methionine into normal and RSV transformed CEF. cpm/mg protein

		Control	L-SAH 1mM	SIBA 0.5mM	SIBA 1mM	A+H 0.5mM-each
Normal Cells	TCA soluble	26140	34300	59480	83330	36000
	nucleic acids	14780	10990	11240	5310	10740
	proteins	290860	236230	175670	73580	273400
	total counts	331780	281520	246390	162220	320140
Transformed Cells	TCA soluble	31640	54920	53780	125550	26350
	nucleic acids	16440	20008	13470	6210	16520
	proteins	202640	247550	121804	39890	183260
	total counts	250720	322478	189004	171650	226130

A = adenosine
H = homocysteine (L)

ter : NaCl 8g, KCl 0.2g, Na_2HPO_4 $2H_2O$ 1,44 g, KH_2PO_4 0.2g, pH : 6.7-7. Radioactivity in the different fractions (acid soluble, nucleic acid and protein) was determined by the method of Schmidt and Thannhauser (12).

L-SAH degradation, was measured by an indirect method : 200 µl of a cell-free extract containing 2-10 mg protein/ml was incubated with 2 mM L-SAH at 37° for different times. The residual concentration of L-SAH in the incubation mixture was estimated from the percentage of inhibition exerted by the mixture on crude tRNA methylases, in comparison with known concentrations of L-SAH incubated in parallel in the same conditions.

Adenosine deaminase (E.C. 3.5.4.4.) was measured in crude extracts of different cell types by determination of the extent of transformation of adenosine to inosine by the change of the absorption at 265 nm as described by Kalckar (13). The adenosine concentration in the reaction mixture was 50 µM. The specific activity is expressed as the nanomoles of adenosine transformed in one hour by 1 mg of protein at room temperature.

L-SAH hydrolase (E.C. 3.3.1.1.). This enzyme catalyses the reversible hydrolysis of L-SAH. L-SAH was measured by the method described by De La Haba and Cantoni (14). The reaction mixture consisted of : 200 µl of cell free extract (protein concentration : 2-10 mg/ml), L-SAH 1mM when hydrolysis was measured and adenosine + L-homocysteine 1 mM each when synthesis was determined. The mixture was incubated at 37° and the reaction stopped by addition of 5 % TCA after various times. Proteins were eliminated by centrifugation and the sample diluted with 100 mM phosphate buffer pH 8.5. The concentration of homocysteine was determined with the reagent 5,5'-dithiobis (2-nitrobenzoic acid) at 412 nm as described by Ellman (15). The specific activity is expressed in nanomoles of homocysteine formed in one hour with 1 mg of protein. Control values due to endogenous SAH concentrations were deduced.

L-amino acid oxidase (E.C. 1.4.3.2.). This enzyme catalyses the oxidative deamination of L-amino acids, in occurrence L-SAH, with formation of γ-thio-α – keto-butyric acid (15). The incubation mixture contains 150 µl L-SAH 2 mM in 50 mM phosphate buffer pH 8.5. The reaction is stopped and freed from protein as above. The keto acids formed react with 3-hydrazinoquinoline at pH 2 at 37°. The absorption of the complex is measured at 305 nm. Sodium pyruvate is used as standard. The specific activity is expressed in ΔA_{305} nm per hour at 37°/mg protein.

TABLE II

Extent of L-SAH degradation by different cell-free extracts*

Cell	Incubation time with 1 mM L-SAH 30 min	60 min	18 hrs
Normal CEF	43	70	74
RSV-transformed CEF	0	14	18
CEF infected for 2 hrs with RSV		86	
24 hrs		12	
48 hrs		12	
72 hrs		16	
CEF infected with RAV	42	73	77

*Results are given in percentage of L-SAH disappeared relative to the concentration at zero time in the incubation mixture.

RESULTS AND DISCUSSION

Methylation of cellular proteins and nucleic acids in whole cells. As shown in Table I incorporation of labelled methyl groups into cellular proteins and nucleic acids is inhibited strongly (60-80 %) in both normal and RSV-transformed cells treated with 1 mM SIBA. Under the same conditions the effect of 1 mM SAH or of the mixture of adenosine and homocysteine (the main degradation products of SAH) is negligible. Furthermore the increase of the radioactivity in the CCl_3CO_2H soluble pool in the presence of SIBA suggests that no major alteration occurs in the cell permeability to methionine in the presence of this drug. The decrease of incorporation of radioactive material into cellular proteins in the presence of SIBA shows that the molecule may inhibit de novo protein synthesis in addition to protein methylation.

Degradation of L-SAH. In order to determine, whether the rapid degradation of SAH could explain its inefficiency as inhibitor in whole cells, the rate and the extent of its degradation was studied in normal, infected and transformed CEF. As shown in Table II, surprisingly, RSV-transformed cells are not able (or only slightly) to degrade L-SAH, while normal cells metabolise it very rapidly. As by the indirect method used one measures the extent of inhibition of a methylase by the residual SAH after incubation, the results in Table II show the unavailability of the inhibitor for the enzyme, due to its degradation and/or modification. As in our experimental conditions the transformed morphology of infected cells becomes apparent on the 4th or 5th day following infection, the

TABLE III

Specific activity of L-SAH hydrolase in normal, infected and transformed cells

Cell type	L-SAH hydrolase activity hydrolysis	synthesis
Normal CEF	32	75
RSV-transformed CEF	18	69
RAV-infected CEF	31	
Bu_{57}-infected CEF at 37°	20	
Bu_{57}-infected CEF at 41°	38	
Normal rabbit cells 905	33	
RSV-transformed rabbit cells A_2	16	
Normal hamster cells $C_{13/15}$	32	
RSV-transformed hamster cells $RS_{2/3}$	20	

Specific activities were determined after 1hr of incubation time under the condition specified in material and methodes.

residual concentration of L-SAH was measured in RSV infected cell extracts at various times post infection and also in extracts of cells infected with a non transforming virus, RAV_1. The initial concentration of L-SAH added was the same in all cases. As shown in Table II, 2 hours after infection RSV infected cells still behave as normal cells but 24 hours later as transformed ones, with respect to SAH degradation and/or modification. Moreover RAV infected cells metabolise or modify SAH as rapidly as normal cells which suggests that the observed phenomenon is connected with the expression of the "SRC" gene and may be used as an early indicator of cell infection by transforming RSV. A similar situation was observed with normal and RSV-transformed hamster and rabbit cell-free extracts : L-SAH degradation or modification proceeded 2-3 times faster in normal than in transformed cells (Table II).

We then investigated whether a change in the intracellular level of SAH hydrolase, L-SAH oxidase or adenosine deaminase, after infection, may explain our observations.

L-SAH hydrolase. Eukaryotes but no prokaryotes possess the enzyme L-SAH hydrolase (16). Furthermore, in all systems tested the equilibrium of the hydrolase reaction favoured synthesis over hydrolysis.

As can be seen from Table III this enzyme is present in normal and in transformed chicken, rabbit and hamster cells. The activity of synthesis is about the same in normal and transformed CEF, whereas the level of hydrolysis is two fold higher in the normal than in the different transformed cells. Unfortunate-

ly, this activity alone is not high enough to explain the observed phenomenon, as the percentage of disappearance of L-SAH due to this enzyme is only 16 in normal and 9 in transformed cells in one hour.

The enzymatic hydrolysis of SAH was measured at different times following infection in cell-free extracts. The decrease of the enzyme level was an early event, the activity in cells 4 to 6 hours after infection being identical to that measured in transformed cells, (results not shown) It seems, however, that the variation in the level of the hydrolase is related to the expression of the "SRC" gene, as the enzyme activity is comparable and higher in normal, RAV-infected and Bu_{57} - infected CEF at 41°, than in CEF transformed by wild type RSV or by Bu_{57} at 37°.

The decreased level of SAH hydrolase is not due to a modification of the Km value for SAH (600 µM and 700 µM respectively with normal and RSV transformed cell-extracts. However, this enzyme can be regulated not only by the presence of the "SRC" gene but also by the substrate SAH or its analogue, SIBA, as they repress the synthesis of the hydrolase by about 60 %. Furthermore, we observed that SIBA is a good inhibitor of this enzyme in vitro, as the Ki values are 700 µM and 800 µM in normal and RSV transformed cells respectively.

L-amino acid oxidase. This enzyme is known to oxidize L-SAH to S-adenosyl-γ-thio-α-keto-butyrate (15). Its specific activity in normal and in transformed cell-free extracts is about the same : 0.16 (expressed as ΔA_{305} nm per hour at 37°/mg protein). The percentage of L-SAH disappearence was estimated to be only 10 after one hour of incubation.

Furthermore L-SAH is not deaminated by commercial adenosine deaminase, by any of the cell-free extracts or by the serum used for the cell culture, at least during an incubation time of 48 hours. However, we observed a 2 to 3 fold higher level of adenosine deaminase in normal cell in comparison to RSV or RAV_1 infected cells. Thus, the drop in the activity of this enzyme appears to be related to infection and not to transformation (unpublished results).

Whether the interesting findings concerning the decrease of the specific activity of adenosine deaminase and SAH-hydrolase in RSV infected cells, and the relation of the latter decrease with the expression of the "SRC" gene can be generalised to other cell-virus system, is now under investigation in our laboratory.

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