Association between the Doubling Time of Various Cells and Tissues, and the SH-Content of Their Soluble Proteins

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Protein-SH-Groups, Tumors, Doubling Time

The number of SH-groups of the soluble proteins (Prot-SH) of 12 different types of animal tumors and of cultivated chicken fibroblasts increases with decreasing doubling time (t_d). Between Prot-SH and t_d an inverse association was found with a significance level of 99.5%.

After 30 min incubation *in vitro*, 4-hydroxypentenal (HPE) reacts with different amounts of the Prot-SH of the investigated cells and tissues. It was found that the absolute, as well as the percental amounts of HPE-reactive Prot-SH increase with decreasing t_d, each of the respective inverse associations was found to be highly significant. The tumors were collected in three groups, each with a corresponding range of t_d and a mean value for Prot-SH. Taking the Prot-SH of the slowest growing tissue (DENA-hepatoma) as the reference value, the increments of Prot-SH were calculated for each of the three groups and were found to be strikingly similar to the number of HPE-reactive Prot-SH. The single Prot-SH increments of the diverse cell and tissue types compared with DENA-hepatoma were found to be highly significantly and inversely associated with the respective Prot-SH decreases caused by HPE.

Hence it may be concluded that HPE reacts preferably with those of the Prot-SH which are of some functional importance for tumor growth and, moreover that the attack on those thiols is the more effective the faster the tumor is growing.

Introduction

Previous papers [1, 2] dealt with the SH-content of soluble cell- and tissue proteins. Furthermore the question was investigated how many of these protein-SH-groups of intact cells and of native tissue slices are reacting with 4-hydroxypentenal (HPE) *in vitro*. HPE is an α,β-unsaturated-γ-hydroxy-aldehyde. Reactive Prot-SH are easily and selectively added to the $C_2 = C_3$ double bond of HPE under the conditions used (30 min incubation in 10^{-5} to 10^{-3} M HPE solution at 37 °C) [3].

Furthermore it could be shown that under the conditions named above HPE does not react with all SH-groups of a given protein but attacks selectively those of high reactivity including thiols of functional importance. For example, from a total of 9 SH-groups of glyceraldehydephosphatedehydrogenase only 3–4, from 15 thiols of aldolase only 1 react with HPE whereby complete loss of enzymatic activity was observed [3a].

Single cells in suspension and native tissue slices of various animal tumors were studied, as well as normal chicken-fibroblasts from a cell culture. The result was: with decreasing doubling time of the cells and tissues, the SH-content of the soluble proteins and the number of HPE-reactive Prot-SH show an increase, respectively [4–6a]. The purpose of the present paper was an extensive statistical analysis of this result.

Material and Methods

Statistical analysis, description of the sample

The SH-content of soluble cell proteins, in terms of μm SH/mg soluble protein (Prot-SH), has been obtained from cells of mice of the NMRI-strain and of rats of the *WAG-Rij*, *Buffalo-* and *Sprague-Dawley*-strains. Since the tumors used in the experiment can only be found in these strains, all the conclusions drawn are valid only for these particular strains. The selection of the animals has been made independently of the characteristics of the variables under consideration. The sample is representative of the population mentioned above and can be considered as a random sample. The population sampled and the target population are therefore equivalent.

Data

The data for all the twelve different tumors (plus chicken-fibroblasts) at hand consist of the doubling time, the PSH-values after incubation in isotone NaCl solution at 37 °C for 30 min and the Prot-SH loss after incubation in isotone NaCl solution for

30 min which contained 5×10^{-3} M HPE (see Table I, columns 9, 2 and 5 resp.).

The incubated cells and tissue slices resp. were homogenized under nitrogen, insoluble components were removed by centrifugation and the supernatant was separated on Sephadex G 25 into a high and a low molecular fraction. In the high molecular fraction the protein concentration was determined by the biuret method and the thiol concentration with 5,5′-dithio-bis-(2-nitro-benzoic acid) (DTNB). For further details see Rindler *et al.* [1], Schauenstein *et al.* [2], Schindler [4]. The results were well reproducible under the conditions used. The doubling times were obtained partly from Schindler [4], partly from Morris *, Goerttler *, and Dostal *. The doubling times are given as pragmatical values without deviations. The Prot-SH-values represent the mean values of three to eleven measurements, obtained from diverse animals.

Statistical method

Since all the data are mean values, and the kind of distribution of the individual measurements is not known exactly, and also the standard deviations of the doubling time are known only in part, we have to use a nonparametric method. In order to establish an association between the doubling time and the SH-content of soluble proteins, we decided to use Kendall's τ-statistic [7, 8].

Results

Implementation of the tests

1. The hypothesis was tested:

H_0: No association exists between the doubling time (days) and the Prot-SH-values; *versus* the alternative

H_1: an inverse association exists.

The doubling times and the SH-contents respectively were ranked as follows: Low SH-content and small doubling time correspond to low ranks, respectively. In Table I the ranks are listed in the usual manner for calculating Kendall's τ.

Therefrom T was calculated to -0.766 with a number of investigated samples $n = 13$. Assuming the validity of H_0, with $n = 13$ a probability $P < 0.005$ was obtained for the test parameter $T = -0.766$. Thus hypothesis H_0 is to be rejected. That

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Table I. SH-content and HPE induced SH-loss of the soluble proteins of cells and tissues with different doubling time.

Tumor	Prot-SH $\pm s_{\bar{x}}$	Rank Prot-SH	Prot-SH increase	$-\Delta$Prot-SH	Rank $-\Delta$Prot-SH	$-\%\Delta$Prot-SH	Rank $-\%\Delta$Prot-SH	t_d	Rank t_d
Ehrlich-Ascites London	0.150 ± 0.008	13	0.088	0.061	12	40.7	10	2	2
Ehrlich-Ascites Heidelberg	0.126 ± 0.004	11	0.064	0.062	13	49.2	13	2	2
Sarcoma 180 Ascites	0.127 ± 0.012	12	0.065	0.051	11	40.2	9	2	2
NK-Ly Ascites	0.121 ± 0.006	10	0.059	0.050	10	41.3	11	2.5	4
Sarcoma 180 Solid	0.118 ± 0.015	9	0.056	0.035	8	21.7	7	4	5.5
Chicken fibroblasts	0.110 ± 0.014	7	0.048	0.047	9	42.7	12	4	5.5
Ehrlich Solid Heidelberg	0.071 ± 0.014	3	0.009	0.024	7	33.8	8	5	7
NK-Ly Solid	0.090 ± 0.009	5	0.028	0.018	4.5	20.0	5	5.2	8
Morris Hepatoma 7800	0.097 ± 0.007	6	0.035	0.015	3	15.5	1	6	9
Yoshida Solid	0.116 ± 0.009	8	0.054	0.018	4.5	15.5	2	6.5	10
Rhabdomyo-Sarcoma	0.072 ± 0.005	4	0.010	0.021	6	29.2	6	7	11
H.-P.-Melanoma	0.062 ± 0.005	1	0.002	0.011	1.5	17.7	4	7.5	12
DENA-Hepatoma	0.064 ± 0.010	2	0.000	0.011	1.5	17.2	3	100	13

Prot-SH, μmol SH per mg soluble proteins; Prot-SH increase, absolute increase of Prot-SH (μmol/mg protein) with respect to the Prot-SH of the tissue with the greatest doubling time (DENA-hepatoma); $-\Delta$Prot-SH, absolute differences between the Prot-SH after incubation in 5×10^{-3} M HPE for 30 min (μmol SH/mg protein); $-\%\Delta$Prot-SH, decrease of Prot-SH in percent; t_d, doubling time (days); $s_{\bar{x}}$, confidence interval of the mean Prot-SH. $\gamma = 0.95$.

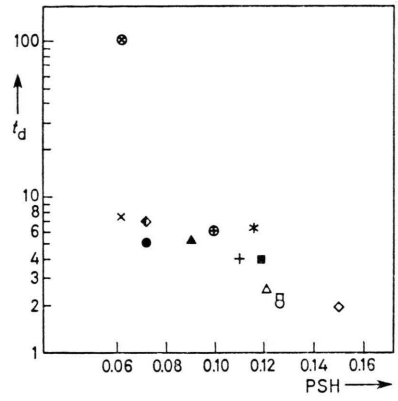

Fig. 1. Association between Prot-SH (PSH = μmol SH/mg Protein) and doubling time.

Tumor	Ascites	Solid	Provenance
Ehrlich, Heidelberg-Lettré	○	●	Prof. Dr. K. Karrer
Ehrlich, London	◇		Prof. Dr. K. Karrer
Yoshida		*	Prof. Dr. K. Karrer
Chicken Fibroblasts		+	Prof. Dr. V. Dostal
			Institut für Krebsforschung, Universität Wien, Austria
Sarcoma 180	□	■	Prof. Dr. G. Bruns
Nemeth-Kellner Lymphosarcoma	△	▲	Prof. Dr. G. Bruns
			Deutsch. Akad. d. Wiss. zu Berlin, Inst. f. Mikrobiologie u. Experiment. Therapie, Jena, GDR
Rhabdomyosarcoma		◆	J. I. M. van Hooft, Ass. Head Biotechnical Dept., Radiobiological Institute T.N.O., Rijswijk, The Netherlands
Harding-Passey Melanoma		×	Prof. Dr. K. Goerttler, Deutsches Krebsforschungszentrum, Inst. f. Experimentelle Pathologie, Heidelberg, GFR
DENA-Hepatoma		⊗	Prof. Dr. G. Ruhenstroth, Max-Planck-Institut für Biochemie, Martinsried bei München, GFR
Morris-Hepatoma 7800		⊕	Prof. Dr. H. P. Morris, Department of Biochemistry, Howard University, Washington, D.C., USA

means the inverse association between doubling time and Prot-SH is significant at least at a 99.5% level (Fig. 1).

2. The same procedure was applied to study the question as to whether there exists an association between Prot-SH after incubation with 0.9% NaCl (Table I, column 2) and the absolute loss of Prot-SH after incubation with HPE ($-\Delta$Prot-SH, see Table I, column 5). The test parameter $T = 0.538$ has a probability $0.005 < P < 0.010$. That means that a direct association exists between the SH-content and the number of HPE-reactive thiols of the soluble cell proteins. This result is significant at a level of at least 99%.

3. The results as mentioned above indicate: Faster dividing cells and growing tissues show greater Prot-SH-values than slower dividing cells and growing tissues. Taking the Prot-SH-value of the slowest DENA-hepatoma as reference, positive differences were found for all other cells and tissues investigated here (Table I, column 4). Furthermore the faster dividing cells and growing tissues lose more Prot-SH after HPE-incubation than slower dividing cells and growing tissues. For the absolute and percental decreases of Prot-SH see Table I, column 5 and 7 resp.

An inverse association was found between the absolute number as well as the percental amount

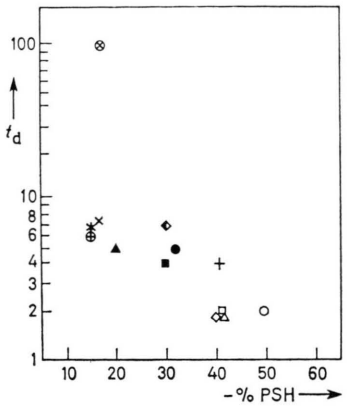

Fig. 2. Association between the percental loss of protein thiols after incubation with HPE and the doubling time.

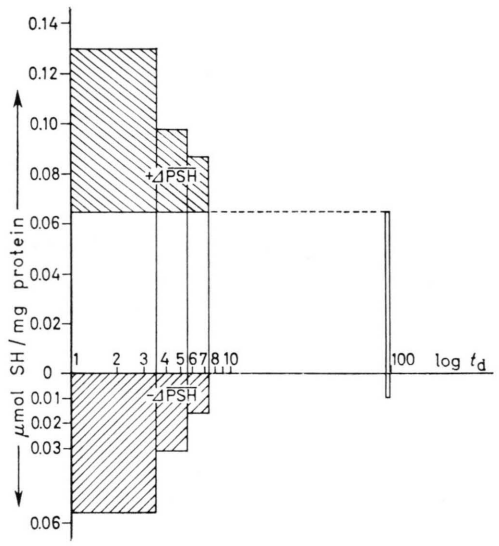

Fig. 3. Classification of various types of cells and tissues according to their doubling time (t_d). The following ranges for the doubling time were chosen (in brackets the corresponding numbers of cell types and tissues) : 1.0−3.5 days (4) ; 3.5−5.5 days (4) ; 5.5−7.5 days (4). For each group the mean Prot-SH values are shown together with the mean Prot-SH increments ($+\Delta\overline{PSH}$) — taking the Prot-SH value of the slowest growing tissue (DENA-hepatoma) as the reference value — and the mean Prot-SH decreases ($-\Delta\overline{PSH}$) caused by HPE

of HPE-reactive Prot-SH and the doubling time. The significance levels were found to be 99.5% for both cases (see Fig. 2).

4. As mentioned above, Prot-SH increments were found for all of the investigated cells and tissues when compared with the DENA-hepatoma. The question was now whether there exists an association between these Prot-SH increments and the Prot-SH decrease, caused by the interaction with HPE. A direct association resulted at a level of at least 99.5%.

The single types of cells and tissues were classified into three groups according to their doubling time; for each group the mean values were calculated for:

a) the Prot-SH-value,

b) the difference between the Prot-SH-value of the respective cells and the Prot-SH-value of the slowest growing tissue (DENA-hepatoma),

c) the absolute loss of Prot-SH induced by HPE.

Fig. 3 demonstrates that the data mentioned under b) and c) are strikingly similar for each group.

5. It was shown above (2.) that the number of HPE-reactive Prot-SH increases with growing Prot-SH-value. If HPE could attack the same percentage of Prot-SH, this would be obvious. Therefore we want to test whether there is an association between loss of PSH in percent, caused by HPE, and Prot-SH-value. The result was a significant direct association of at least 95%.

Discussion

In the pertinent literature [9, 9a] we often find theories and hypotheses that certain SH-groups of specific cell proteins are involved with cell division. As far as we know, this paper proves for the first time that the inverse association between the SH-content of the soluble proteins and the doubling time of several rapidly growing tumors ($t_d < 8$ days) is highly significant. One possible interpretation of this result may be given by the findings of Apffel *et al.* [10], according to which the activity of a NADPH-dependent protein-disulfide reductase is consistently much higher in tumors than in normal tissues.

Further results concern the action mechanism of HPE. The biochemical and biological effects of HPE are caused solely by a reaction with various SH-groups [6, 11, 12]. By the action of HPE the total soluble proteins lose increasing numbers of SH-groups with increasing SH-content. Also the percentage of HPE-sensitive SH-groups increases with increasing total Prot-SH-value of the cell proteins.

Since the total SH-content of soluble cell proteins increases with the growth rate, it was to be

expected that faster dividing cells contain absolutely and relatively higher portions of HPE-reactive SH-groups in the soluble proteins. The corresponding associations were found to be significant at a 99.5% level for both cases.

After it has been proven that the total Prot-SH value is associated with the doubling time, this must hold also for the Prot-SH-increase, taking the Prot-SH-value of the slowest growing tissue (DENA-hepatoma) as the reference value (see Fig. 3).

Hence it may be concluded that these additional Prot-SH are of some functional importance for the growth rate.

With significance of 99.5%, the μmol of HPE-sensitive Prot-SH are inversely associated with the doubling time and, moreover, are strikingly resembling the μmol of additional Prot-SH mentioned above. This suggests that HPE preferably attacks those protein thiols being of functional importance for the cell division and that the attack is the more effective the higher the division rate is.

Most probably the findings discussed in this paper are not only restricted to tumor cells but may be of general validity as indicated by the results with cultivated normal chicken fibroblasts.

Possibly it may be argued that the associations found in this paper might be the so-called "non-sense-associations". Therefore it seems to be necessary to point out:

The initial model conceptions used to the statistical analysis presented here are based on experimentally proved facts, namely:

a) The DNA-synthetase has SH-groups being of functional importance for the activity of this enzyme system and the activity influences directly and positively the cell division.

b) There exist acidic residual proteins bound to the DNA which contain SH-groups and stimulate the biosynthesis of nucleic acids.

c) Several authors (*e. g.* Szent-Györgyi [9a], Együd [9a], Mazia [9], Ord [9], Rapkine [9], Wang [9], Hilton [9], Stocken [9], have postulated a direct and positive association between SH-groups of various cellular proteins and cell division.

In view of these concepts a "non-sense-association" seems to be out of question.

One of the authors, E. Schauenstein, wishes to express his gratitude to the persons and institutions named in the legend to Fig. 1 for the generous supply of tumor samples.

The data concerning the hepatoma 7800 have been taken from an extensive investigation which will be published in full detail together with Professor H. P. Morris in a separate paper.

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