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OXYGEN DEPENDENT REGULATION OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE *IN VIVO* AND POSSIBLE SIGNIFICANCE FOR REPLICON INITIATION

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SUMMARY: The intracellular concentration of the M2 specific free tyrosyl radical of ribonucleotide reductase in cultured Ehrlich ascites cells was estimated by EPR spectroscopy during imposition and after reversal of hypoxia. Under the same conditions, the intracellular intensity of CDP reduction was estimated indirectly by measuring the incorporation of radioactivity into DNA from labeled ribo- and deoxyribo-cytidine, respectively. The radical concentration distinctly decreased under hypoxia and reincreased upon reaeration. At the same time, the CDP reduction was greatly diminished and reactivated, respectively. These observations are interpreted in the sense of an O_2 dependent regulation of the intracellular activity of ribonucleotide reductase. The O_2 dependent deactivation and reactivation of the enzyme was temporally related to a specific suppression or a burst-like release of replicon initiations, respectively. Addition of 100 µM dCtd to hypoxic cells could substitute for reoxygenation with respect to triggering replicon initiations. A possible implication of the intracellular ribonucleotide reductase activity and the size of the dCTP pool in replicon initiation is dicussed.

The active form of mammalian ribonucleotide reductase contains, in its M2 subunit, a tyrosyl free radical (1-5). Generation and maintenance of this radical in enzyme preparations was reported to require O_2 (1-4). An EPR signal identifying this radical was shown to be detectable even in whole mammalian cells (1,6). It was proposed that introduction resp. destruction of the free radical may represent a way of regulating reductase activity *in vivo* (2-4).

Ehrlich ascites cells and several other mammalian cell lines are capable of regulating the course of replication of their genome in dependence on the O_2-tension in their environment (7-9). This regulation concerns cycling cells generally committed to DNA synthesis and **does not** (8,9) cause switching between a cycling and a resting state (G_0) as do cell specific proliferation signals. At low O_2-tension, replicon initiations are specifically, reversibly, and coordinately suppressed whereas DNA chain growth and maturation in already initiated replicons continue essentially normally. Reelevating the pO_2 triggers a burst of scheduled initiations without detectable overreplication, i. e. repeated initiation of the same replicons within a single S-phase (10). The shutdown of scheduled

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initiation events is possible at various stages of the S-phase, even before the activation of the very first replicons (8,9,11). In the latter case, the cells arrest near the G_1/S border in a state definitively different from G_0 (3) as they are ready to activate normal S-phase replication within a few minutes upon reoxygenation (8,11).

The initiation of new rounds of replication of Polyoma DNA seems to be dependent on an intact supply of dCTP (12). Impulse cytofluorometric data indicate that an anaerobic arrest of Ehrlich ascites cells at the G_1/S border is specifically abolished by addition of dCtd (13). As mentioned, we regard this cell cycle arrest as one of the manifestations of the O_2 dependent regulation of replicon initiation (8,9,11).

Here, we demonstrate that intracellular deradicalisation or reradicalisation of the tyrosyl residue in the active center of ribonucleotide reductase occurs in living Ehrlich ascites cells when hypoxia of "physiological" stringency is established or abolished, respectively. The extent of intracellular CDP reduction follows these changes which are temporally related to the suppression or retriggering of replicon initiations, respectively. Supplementing hypoxic cells with 100 µM dCtd and thereby reelevating the intracellular dCTP level diminished under hypoxia (13) can substitute for reoxygenation in triggering replicon initiations. These observations stimulated us to discuss the possibility of causal relations between the imposed changes of the pO_2 and the observed changes of ribonucleotide reductase activity and of dCTP pool size on the one side and of the replicon initiation frequency on the other side.

Methods

Descriptions of the Ehrlich ascites cells used, the hypoxic standard cell culture, the radioactive labeling procedures and the analysis of the length distributions of replicative daughter strand DNA by alkaline sedimentation are given in (7).
For EPR analyses 200 ml cell culture samples (1 - 1.2 x 10^8 cells) were transferred into 1.5 vol. of ice-cold PBS (150 mM NaCl, 15 mM phosphate, pH 7.2) in pre-cooled large glass centrifuge tubes and centrifuged at 2- 5 °C for 6 min. at 800 x g. The sediment was suspended in about 0.5 ml cold PBS (final volume 1-1.2 ml), transferred to quartz EPR tubes (O 4 mm) and centrifuged for 5 min. at 1 600 x g. Supernatant and cell sediment exceeding 3 cm above the bottom (maximal height measured in the EPR spectrometer) were discarded. After freezing in liquid N_2 the samples were stored at 77 °K until measured. When hypoxic cells were analysed, all operations were performed under a protective atmosphere consisting of the gassing mixture for hypoxia or pure argon, and the PBS used was equilibrated with the gassing mixture after boiling. EPR spectra were recorded at 77 °K in a Bruker ESP 300 X-Band spectrometer using a suitable cold finger Dewar vessel containing liquid N_2. Modulation frequency was 100 kHz. The spectra presented were obtained each by 10 scans without further corrections and plotted at identical scale.
For measuring the radioactivity incorporated from [5-^{3}H]Ctd resp. [5-^{3}H]dCtd (specific activity 21 Ci/mM resp. 23 Ci/mM, supplied by Amersham-Buchler, Braunschweig, FRG) into DNA, the cell samples were washed twice with 10 ml of ice-cold Hanks solution, extracted twice for 10 min. at 0 °C with 5 ml 5 % TCA, dissolved in 1.5 ml 0.3 M NaOH, incubated overnight at 25 °C, precipitated with 2 ml 15 % TCA, collected on Whatman GF/C filters (O 22 mm), washed 3 times on the filter with 10 ml 1% TCA, dryed and counted in toluene based scintillation fluid. This procedure removed all radioactivity primarily acid soluble and all acid precipitable radioactivity present in form of RNA. No loss of ^{14}C prelabel (DNA) occurred.

Results and Discussion

In order to allow reference to our prior work with hypoxic cells (7-11,14) we strictly followed our standard protocol of "transient controlled hypoxia" (8). The latter consists of equilibrating the cell cultures with an argon/5% CO_2 mixture containing 200 ppm O_2 and reoxygenating them, after 5 or 10 h, by addition of 1/4 vol. of medium saturated with pure O_2. Thereby, the pO_2 takes 30 - 60 min to reach 3.5 -1.7 % of the atmospheric tension where the suppression of replicon initiations emerges (8,9). Reoxygenation occurs at once. The 200 ppm O_2 component in the gas mixture precludes extremely hypoxic conditions which cause cell damage and severely affect the reversibility of the shutdown of replicon initiation (8). As outlined in detail in (8), these conditions, called by us "controlled hypoxia", are tought to occur intermittently in an Ehrlich ascites tumor growing *in vivo* and very probably represent a specific kind of "physiological" environment of animal cells in regions of poor vascularisation, e.g. in certain regions of solid tumors.

Fig. 1 shows results of EPR studies on the free radical of the M2 subunit of ribonucleotide reductase in cultured Ehrlich ascites cells which were frozen live in liquid N_2. To ensure the identity of the signal we confirmed that it disappeared after hydroxyurea treatment (1) and changed from a doublet to a singlet structure after interchanging ^{1}H-L-tyrosine and DL[ß,ß$^2H_2$]-tyrosine in the medium (6) (Fig. 1 A-D). Then, we studied the signal under and after reversal of controlled hypoxia. Fig. 1 E - K shows the signals recorded with cell samples obtained in a standard transient hypoxia experiment. The observed effects qualitatively reflect the kinetics of the pO_2 in the medium reported earlier (8).

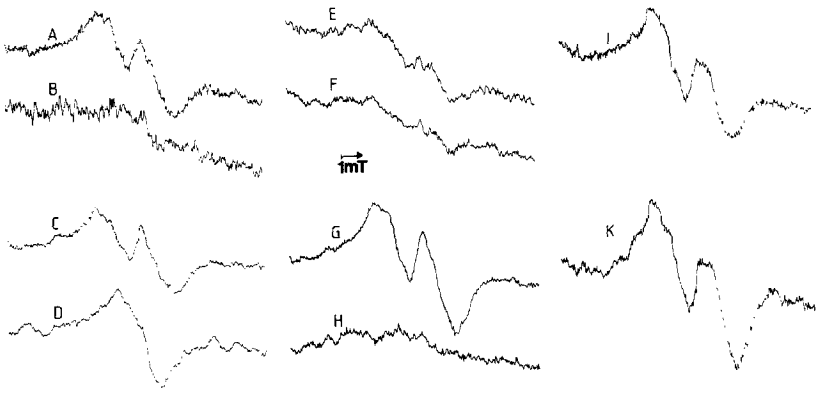

Fig.1 EPR spectroscopy of the tyrosine free radical of the M2 subunit of ribonucleotide reductase in cells frozen live in liquid N_2: Influence of hydroxyurea (A,B), of growth in presence of [ß,ß$^2H_2$]-tyrosine (C,D) and of transient controlled hypoxia (E - K). For A,B a cell culture was divided and one half (B) was incubated with 3 mM hydroxyurea for 20 min. For C,D a 400 ml cell culture was established in medium lacking tyrosine and then divided. One half was supplied with 40 µM normal L-tyrosine (C), the other (D) with 80 µM DL[ß,ß-$^2H_2$]-tyrosine (Reagenta, Uppsala, Sweden). Both cultures were analysed after 30 h (cell number triplicated). E - K, portions of a large cell culture were incubated either aerobically for 5 h (G) or under controlled hypoxia for 1, 2, and 5 h (E,F,H), or reoxygenated after 5 h hypoxia and further incubated under air for 10 and 40 min.(I,K). The samples were stopped and processed for EPR spectroscopy as described in Methods.

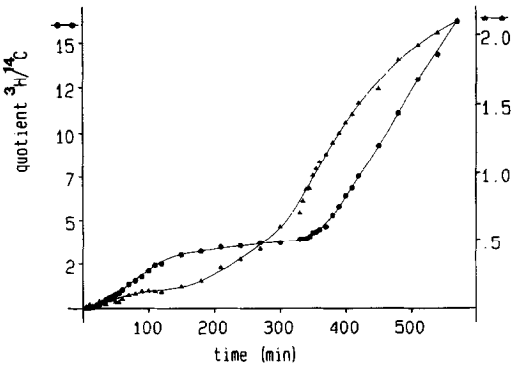

Fig.2 Influence of transient (5 h) controlled hypoxia on the incorporation of [^{3}H]ribo-Ctd (●) and [^{3}H]dCtd (▲) into cellular DNA (alkali stable, acid precipitable cell fraction). A cell culture, prelabeled with [2-^{14}C]dThd in the preceding passage, was supplemented with 50 µM ribo-Ctd and dCtd. After 2 h, the culture was divided and 2 µCi/ml of [5-^{3}H]ribo-Ctd were added to one part and 2 µCi/ml of [5-^{3}H]dCtd to the other (time=0). 30 min. later hypoxic gassing was started, followed by reoxygenation after 5 h. At times indicated, 1 ml samples were withdrawn and processed as described in Methods. The results were normalized to the ^{14}C-prelabel of the samples (about 20 000 cpm).

Within 2 h, the signal decreased below the noise level. Reoxygenation caused its fast restoration. Already 10 min after resupplying O_2 (Fig. 1I) 3/4 of the control (Fig. 1G) were attained. The signal to noise ratio of the control indicates that hypoxia reduced the radical concentration by a factor of 5 at least. A complete abolition of the free radical is not assumed (see below).

To examine the intracellular activity of ribonucleotide reductase in a transient hypoxia experiment, we chose an indirect way: Salvage routes of the dCTP pool supporting DNA synthesis can start with either ribo-Ctd and/or dCtd (3,4). Normally, the first route passing through ribonucleotide reductase prevails in mammalian cells. However, we expected an increased usage of the second route if CDP reduction is diminished under controlled hypoxia. Therefore, we examined the influence of transient hypoxia on the utilisation of both nucleosides for the synthesis of DNA (measured as alkali stable - acid insoluble material, Fig. 2). In this experiment we added 50 µM ribo-Ctd and dCtd to either incubation thus providing, (i) by dCtd, progress through the S-phase despite of hypoxia (13), and (ii) by both nucleosides, a constant "pressure" on both salvage pathways. Thereby, we had to put up with a pool expansion. The latter delayed the initial equilibration of intracellular dCTP with the radioactive tracers (confirmed in a control experiment, data not presented) and also the detectability of a change of the main salvage supply from ribo-Ctd to dCtd or vice versa. Nevertheless, Fig. 2 demonstrates that the [^{3}H]-ribo-Ctd incorporation rate distinctly decreased upon deoxygenation and then attained a steady state at a rate significantly different from zero, indicating the persistence of a basic CDP reduction. About 70 min after reoxygenation a new steady state was attained . The slope of the curve indicates a 16-fold increased utilization of ribo-Ctd relative to the hypoxic state. The [^{3}H]dCtd incorporation rate behaved inversely. However, an obviously considerably expanded pool of dCtd derivatives, perhaps partly consisting of deoxyliponucleotides (15), allowed no steady state within the observation period. Nevertheless, the result is consistent with a supposed substitution

of the external dCtd for a diminished CDP reduction under hypoxia. As the pO_2 is the only parameter changed in this experiment, we conclude that the intracellular activity of ribonucleotide reductase, at least with respect to CDP as substrate, is tuned according to the O_2 tension in the medium. Most probably, this tuning occurs by changing the intracellular concentration of the M2 specific tyrosyl radical which can be observed under the same conditions (Fig. 1). As dATP, dGTP and dTTP, in contrast to dCTP, exert strong allosteric effects on the enzyme (3,4), analogous experiments using the corresponding ribo- and deoxyribonucleosides are impossible. Perhaps, just this complicated feedback regulation allows only minor changes of the non-cytosine dNTP's when the amount of active enzyme in living cells varies (see below).

Our findings support the proposal of a regulatory role of radicalisation/deradicalisation of the tyrosyl residue in the active center of ribonucleotide reductase (2-4). The physiological significance of this regulation remains obscure. However, preceding work (7-9,14) of our laboratory yielded compelling evidence, in particular by DNA fiber autoradiography and by analysis of the length distributions of replicative daughter strand DNA, that exactly the same transient hypoxia protocol as used in the above experiments caused, during the hypoxic period, a progressive specific suppression of replicon initiations followed

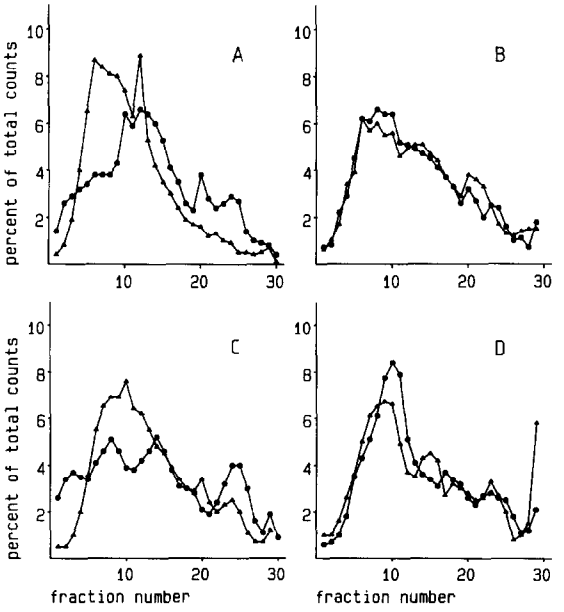

Fig.3 Effect of dCtd on hypoxic cells: Changes produced by reaeration resp. addition of 100 µM dCtd in the alkaline sedimentation profiles of growing daughter strand DNA of hypoxic cells. A cell culture was divided into 8 parts 2 h after transfer to fresh medium. A, (▲) 5h normoxia - normoxic labeling (control), (●) 5h hypoxia - hypoxic labeling. B, (▲) 5h hypoxia - 40 min hypoxia in presence of 100 µM dCtd - hypoxic labeling, (●) 5h hypoxia - 40 min normoxia - normoxic labeling. C,D, same arrangement as A,B, except that the duration of the aerated resp. hypoxic preincubation was 10h. Sedimentation was from left to right. Cells were pulse labeled (8 min.) lysed on the gradients and sedimented as described (14). ^{14}C Lambda DNA, used as an internal sedimentation marker, was reproducibly recovered in fraction 9 (37-41 S). The total ^{3}H-cpm recovered from the gradients varied between 13 500 (C,●) and 63 600 (A,▲).

by a burst-like reappearance of initiations upon reoxygenation. Resuming a former supposition of P. Reichard (12) we looked for a link between ribonucleotide reductase and replicon initiation. Stimulated by observations of Löffler et al. (13) as well, we compared, in a modified standard transient hypoxia experiment, the effectiveness of reoxygenation with that of the addition of dCtd for triggering replicon intitiations in hypoxic cells by analysing the length distributions of growing daughter strand DNA by alkaline sedimentation. Reoxygenation was simply substituted by addition of 100 μM dCtd in parallel cell samples. Fig. 3 demonstrates that the well known progressive depletion of recently initiated short chains occurring under hypoxia (7-9,14) is reverted equally well by dCtd as by reoxygenation.

Since the dCtd addition is suitable to elevate effectively the intracellular dCTP level (4) whis is decreased under hypoxia (13) it seems worth to dicuss (i) wether it may be possible that the dCTP pool size primarily depends on the amount of active M2 present while the other 3 dNTP pools are held approximately constant by feedback regulation as long the reductase activity does not fall below a certain critical level and (ii) whether a causal relation could exist between the size of the dCTP pool and the frequency of replicon initiations by which the rate of DNA synthesis is determined in the first place (16). No contradictory data are known to us. Compatible observations were published (12,17,18). The expression of the M2 subunit is cell cycle regulated (17). During the cell cycle of CHO cells Leeds et al. (18) found an almost parallel course of the activity of ribonucleotide reductase, of the size of the dCTP pool and of the rate of DNA synthesis. The maximum in S differed from the minimum in G_1 by about one order of magnitude. However, the corresponding changes of dATP, dGTP and dTTP were comparatively moderate and the minimum of the nuclear dCTP level in G_1 remained distinctly above the K_M of replicative polymerase for the nucleotide (18) thus fullfilling the prerequisites for DNA chain elongation, e.g. for DNA repair, in all phases of the cell cycle. Consistently, in contrast to replicon initiation, DNA chain elongation is largely normal under controlled hypoxia (7). Further studies, in particular on the sizes of all 4 dNTP pools in transient hypoxia experiments, may help to clarify the questions raised.

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