Counteraction of pRb-dependent protection after extreme hypoxia by elevated ribonucleotide reductase

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Abstract. We have studied hypoxia-induced cell cycle arrest in human cells where the retinoblastoma tumour suppressor protein (pRb) is either functional (T-47D and T-47DHU-res cells) or abrogated by expression of the HPV18 E7 oncoprotein (NH1K 3025 cells). We have previously found that pRb is dephosphorylated and rebound in the nucleus in T-47D cells arrested in S-phase during hypoxia and that this binding is protracted even following re-oxygenation. In the present study, however, we show that the long-lasting arrest following re-oxygenation induced by pRb-binding in the cell nuclei may be overruled by an elevated level of ribonucleotide reductase (RNR). This seems to create a forced DNA-synthesis, uncoordinated with cell division, which induces endoreduplication of the DNA. The data indicate that the cells initiating endoreduplication continue DNA-synthesis until all DNA is replicated once and then may start cycling and cell division with a doubled DNA-content. Corresponding data on the pRb-incompetent NH1K 3025-cells show similar endoreduplication in these. Thus, the data indicate that endoreduplication of DNA following re-oxygenation may come, either as a result of hypoxic arrest of DNA-synthesis when pRb-function is absent in the cells, or if it is overruled by increased RNR. The present study further shows that pRb not only protects the culture by arresting most of the cells that are exposed to extreme hypoxia in S-phase, but also increases cell survival by means of increased clonogenic ability of these cells. Interestingly, however, cells having an elevated level of RNR have equally high survival as wild-type cells following 20 h extreme hypoxia. If RNR-overruling of pRb-mediated arrest following re-oxygenation results in an unstable genome, this may therefore represent a danger of oncogenic selection as the protective effect of pRb on cell survival seems to be maintained.

INTRODUCTION

Hypoxia is a common environmental stress that is known to be an important component of many physiological and pathological conditions. In cancers there may be small or large areas of severe hypoxia, which may be chronic because of abnormalities in the development of the capillary network in such tumours. But even in most normal tissues, oxygen tension varies over time and acute hypoxia appears regularly as a result of transient occlusion of small vessels.

The presence of hypoxic microenvironments in tumours seems to promote development of aggressive phenotypic traits (Kim *et al.* 1997; Wouters *et al.* 2003). It has been suggested that hypoxia provides a physiological pressure in tumours, selecting for cell subpopulations with a survival and growth advantage and an increased metastatic potential (reviewed by Rofstad 2000). In order to avoid both unnecessary cell damage due to hypoxia, and repopulation of cells already damaged by hypoxia, regulation of cell cycling and of cell death under various low levels of hypoxia must be vital and has also been extensively studied during recent years (Åmellem *et al.* 1996, 1998; Green & Giaccia 1998).

Cells are particularly sensitive to damage by hypoxia while in S-phase. The immediate response of mammalian cells to severe hypoxia is thus to turn off DNA synthesis. The sensing system for this process seems to be deactivation of the two oxygen-dependent enzymes dihydro-orotate dehydrogenase and ribonucleotide reductase (RNR), resulting in complete cessation of de novo synthesis of pyrimidine deoxyribonucleotides (Löffler 1987; Probst *et al.* 1989; Åmellem *et al.* 1994). We have, however, previously shown that the retinoblastoma protein (pRb), which under normal conditions controls passage through a G₁-restriction point in the cell cycle, plays an extensive role under hypoxic conditions, where pRb is activated even in S-phase (Åmellem *et al.* 1996). Thus, pRb seems to represent a guarantee that cells exposed for several hours to hypoxic conditions while in S-phase are prohibited from further cell cycling. This may represent an important protection against disadvantageous proliferation of damaged cells following hypoxia while in S-phase, and our conclusion has been that pRb overruled even the deoxyribonucleotide-supply mechanism of arrest as it maintained the prohibition of DNA synthesis even after re-oxygenation (Åmellem *et al.* 1996).

In a recent study, however, we have shown that the pRb-induced cell-cycle arrest might still be overruled by ribonucleotide reductase, but only if the cells expressed the enzyme at a highly elevated level (Graff *et al.* 2002). In that study, cells were treated with mild hypoxic conditions of 1300 p.p.m. O₂, which is a level of O₂-tension where cell respiration is not affected by oxygen shortage (Froese 1962; Boag 1970). However, under these conditions, DNA-synthesis is completely inhibited during hypoxia primarily because of the lack of activity of RNR, the essential last step operator in the production of pyrimidine deoxyribonucleotides (Eklund *et al.* 2001). Thus, by this oxygen-sensing mechanism, the cells have an elegant way of immediately preventing DNA-synthesis when the O₂-tension suddenly drops to a low level, even though that level is still high enough for normal cellular respiration. Only if hypoxia lasts for many hours is pRb dephosphorylated and thus takes over the control of the cell cycle (Åmellem *et al.* 1996, 1998).

In the present study, we have investigated whether hypoxia-induced pRb-control of DNA synthesis could be overruled by elevated ribonucleotide reductase even if hypoxia is made as severe as to abolish respiration, and furthermore we have tested whether this influences cell survival following hypoxia. Cells of three types have been treated with extremely hypoxic conditions (i.e. < 4 p.p.m. O₂) where cell respiration was severely hampered (Froese 1962). We

have used T-47D cells having a normal pRb function, NHIK 3025 cells having defective pRb and T-47DHU-res cells which seem to have a normal pRb function, but at the same time a highly elevated level of RNR as a result of adaptation to high levels of hydroxyurea (Graff *et al.* 2002). We have measured the nuclear binding of pRb in relation to the cell cycle both during hypoxic treatment and after re-oxygenation. The findings indicate that pRb prevents S-phase cells from continuing undue cell-cycle progression following re-oxygenation and also prevents the cells from endoreduplicating their DNA, abrogating the start of a new round of DNA replication without first completing mitosis. The protection against DNA endoreduplication, however, seems to be overrun by an elevated level of RNR. Our results also indicate that the presence of pRb may represent protection with respect to survival for cells arrested for 20 h by extreme hypoxia in S- or G₁-phase. Interestingly, we find that this is not affected by an increased level of RNR.

MATERIALS AND METHODS

Cell cultures

Cells of the human breast cancer cell line T-47D (Keydar *et al.* 1979) were grown as monolayer cultures in RPMI 1640 medium (Gibco, Paisley, UK), supplemented with 10% foetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 200 units/l insulin and 1% penicillin/streptomycin (Gibco). The doubling time for T-47D cells was 37 ± 2 h (Stokke *et al.* 1993). Cells of the human hydroxyurea-adapted cell line T-47DHU-res (Graff *et al.* 2002) were grown as monolayer cultures in RPMI 1640 medium (Gibco), supplemented with 10% foetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 200 units/l insulin, 1% penicillin/streptomycin (Gibco) and 0.5 mM hydroxyurea. Cells of the human cervical carcinoma cell line NHIK 3025 (Oftebro & Nordbye 1969) were grown as monolayer cultures in MEM medium, supplemented with 15% foetal calf serum (Gibco), 2 mM L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). The doubling time for NHIK 3025 cells was 18 h (Koritzinsky *et al.* 1998). NHIK 3025 cells contain the human papillomavirus 18 (HPV18) (Åmellem *et al.* 1998). Cell cultures were kept in exponential growth at 37 °C in air containing 5% CO₂ by re-culturing two times a week.

Hypoxic cell cultures

The technique of introducing and maintaining various hypoxic conditions in cell cultures has been described previously (Pettersen & Lindmo 1981). Briefly, the cells were seeded in 70-mm glass dishes (Anumbra, Prague, Czech Republic) one day before the experiment and incubated in a CO₂ incubator. At the appropriate time, the glass dishes were brought from the CO₂ incubator into a walk-in incubator room at 37 °C. De-oxygenation took place by continuous flushing of the chamber with a gas mixture (Hydro Gas, Oslo, Norway) of 97% N₂, 3% CO₂ and < 4 p.p.m. O₂ at 37 °C using the set-up described earlier (Løvhaug *et al.* 1977). The final level of O₂-concentration in the chamber was established about 12 min after start of flushing. Untreated control populations were kept in the CO₂ incubator during the experiment.

Extraction, fixation and staining for measuring contents of DNA, BrdUrd and nuclear bound pRb

All steps were carried out at 0 °C. Trypsinized cells were washed once in phosphate-buffered saline (PBS). For detection of nuclear bound pRb, cells were prepared by re-suspending in 1.5 ml low-salt detergent buffer (10 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulphonyl

fluoride, 0.1% Nonidet P-40, 10 mM phosphate buffer (pH 7.4)). After 10 min, the extracted cells, hereafter termed 'nuclei', were supplied with 0.5 ml 4% paraformaldehyde. Nuclei were fixed for 1 h, and then washed twice in washing buffer [PBS with 0.1% Triton X-100 (pH 7.4)]. The presence of pRb in the cells was detected using the G3-245 monoclonal antibody (Pharmingen, San Diego, CA, USA). As secondary antibody biotinylated horse anti-mouse IgG₁ (HAM) (Vector Laboratories, Burlingame, CA, USA) was used and detected with streptavidin-FITC (Amersham Biosciences, Little Chalfont, UK). DNA staining was performed with 2 µg/ml Hoechst 33258. Pulse-chase labelling with bromodeoxyuridine (BrdUrd) was used to record DNA synthesis in cells under hypoxic conditions. Cells were incubated with medium supplemented with 35 µM BrdUrd for 30 min and washed twice in medium before the hypoxic treatment. Harvested cells were washed once with PBS, fixed in 70% methanol, and stored at -20 °C. Fixed cells were washed with PBS, re-suspended in 2 ml of 0.2% pepsin in 2 N HCl, and incubated for 1 h at room temperature (22 °C). The cells were washed three times in PBS, and a three-layer procedure for staining BrdUrd was employed. Cells were re-suspended in 50 µl anti-BrdUrd antibody (Becton-Dickinson, Franklin Lakes, NJ, USA). Further procedures were performed as for detection of pRb.

Flow cytometry

Stained cells were measured in a FACStar^{PLUS} flow cytometer (Becton Dickinson) equipped with one argon and one krypton laser (Spectra Physics, CA, USA) tuned to 488 nm and UV, respectively. The following parameters were measured: forward light scatter (FSC), side scatter (SSC), FITC fluorescence intensity (pRb and BrdUrd), integrated Hoechst 33258 intensity (DNA content), Hoechst 33258 fluorescence pulse height, and Hoechst 33258 fluorescence pulse width. The data were gated on FSC versus SSC and Hoechst 33258 fluorescence pulse area versus pulse width to exclude debris and aggregates of cells, respectively (not shown in the figures). The green fluorescence intensities were calibrated with fluorescent beads prior to each experiment such that the FITC fluorescence intensity measured in different experiments could be compared.

Cell synchronizing and survival analysis

Cells containing G₁- or S-phase quantities of DNA were sorted by use of a FACStar^{PLUS} flow cytometer. The cells were incubated for 20 min with 8 µM Hoechst 33342 prior to sorting. After sorting, S-phase and G₁-phase cells were seeded on glass dishes and incubated in a CO₂-incubator until the cells had attached to the bottom. Thereafter, half of the dishes were exposed to hypoxia for 20 h before they were returned to the CO₂ incubator for colony formation. The other half of the dishes were left untreated in the CO₂-incubator as aerobic controls.

Western blotting

Cells were lysed with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and proteins were separated on an 8% SDS-polyacrylamide gel with a 4% stacking gel. The proteins were transferred onto Hybond-P (Amersham Biosciences) nitrocellulose membrane using Mini Trans Blot (Bio-Rad) tank blotting with blotting buffer containing 2.5 mM Tris (pH 8.3), 19.2 mM glycine and 20% methanol. The membranes were blocked at 4 °C overnight in TBS containing 5% non-fat dry milk and 0.1% Tween-20, before immunolabelling with 1 µg/ml polyclonal antibodies against either p53R2 or R2 (supplied by Hirofumi Arakawa). The secondary antibody (peroxidase conjugated goat anti-rabbit) was supplied by Dako (Glostrup, Denmark). Detection of bound antibodies was performed with ECL (Amersham). Equal quantities of cells were loaded of each sample.

Figure 1. Two-parametric DNA versus pRb histograms of nuclei extracted from T-47D- (a–c), NHIK 3025- (d–e) and T47DHU-res-cells (f–h). The histograms, respectively, represent exponentially growing aerobic control cells (a, d, f), cells exposed to extremely hypoxic conditions (< 4 p.p.m. O_2) for 18 h (b, g) and cells first treated with the hypoxic conditions for 18 h, then re-oxygenated and grown under aerobic conditions for 6 h (c, h). Nuclei were stained with FITC-binding to pRb and with Hoechst 33258-binding to DNA, except histogram (e) for which the primary antibody had not been included. Data were analysed with the PC-Lysis (Beckton-Dickinson) program.

RESULTS

Correlation between nuclear binding of pRb and the proliferation status of cells in S-phase following re-oxygenation

To follow how the status of binding pRb to the nucleus correlated to the cell cycle, we performed flow cytometric studies on the nuclei. Binding of pRb to the nuclei is indicated by boxes (Fig. 1). In control cells of both T-47D- and T-47DHU-res-type pRb was found to be de-phosphorylated and bound to the nucleus in early G_1 , as seen from the pRb-positive (i.e. pRb⁺-marked) sub-population in G_1 in both panels A and F of Fig. 1. In NHIK 3025 cells, no such binding was seen when comparing cells analysed both with and without the primary antibody against pRb (Fig. 1d and e), which was expected as these cells lack pRb (Stokke *et al.* 1993). In S-phase cells of both T-47D and T-47DHU-res-type pRb was activated (Fig. 1b and g) as expected from previous studies (Åmellem *et al.* 1996; Graff *et al.* 2002). Following re-oxygenation, pRb continued to remain bound to the nucleus for the first 6 h, indicating the protracted nature of this nuclear binding of pRb (Fig. 1c and h).

Figure 2. Two-parametric DNA versus BrdUrd histograms for T-47D cells (a, d, g) and the corresponding one-parametric DNA-histograms of cells that had not incorporated BrdUrd (b, e, h) or cells that had incorporated BrdUrd (c, f, i). The three two-parametric histograms, respectively, represent exponentially growing aerobic control cells (a, b, c), cells exposed for 18 h to extremely hypoxic conditions (< 4 p.p.m. O_2) (d, e, f) and cells first treated with hypoxic conditions for 18 h, then re-oxygenated and grown under aerobic conditions for 30 h (g, h, i). The cells were labelled with BrdUrd for 30 min under aerobic conditions, then washed and fixed either immediately (a, b, c), immediately following the hypoxic treatment (d, e, f) or after re-oxygenation and additional 30 h under aerobic conditions (g, h, i). BrdUrd-labelled cells were FITC-stained with a three-layer procedure, whereas DNA was stained with Hoechst 33258. BrdUrd and DNA contents were measured by flow cytometry as described in MATERIALS AND METHODS. The DNA histograms from the BrdUrd⁻ (b, e, h) and BrdUrd⁺ (c, f, i) fractions were obtained by gating as indicated by the windows in histograms a, d and g. The data was analysed with PC-Lysis.

Comparison of DNA synthesis in cells after re-oxygenation

Only DNA-replicating cells incorporate BrdUrd into their DNA (referred to as BrdUrd⁺ cells) and are thus easily distinguished from non-replicating (BrdUrd⁻) cells by 2-parametric flow cytometry (Fig. 2). In a pulse-chase experiment, exponentially growing T-47D cells were labelled with BrdUrd for a 30 min-pulse before an 18-h exposure to extremely hypoxic conditions followed by re-oxygenation and further growth under aerobic conditions for 30 h (Fig. 2). The experiment clearly showed that all cells labelled with BrdUrd (BrdUrd⁺) before the hypoxic

NH1K 3025 cells

Figure 3. DNA-histograms of BrdUrd⁺ T-47D-cells (a, b), NH1K 3025-cells (c, d) and T-47DHU-res-cells (e, f) labelled with BrdUrd for 30 min under aerobic conditions and thereafter either exposed for 18 h to extremely hypoxic conditions (a, c, e) or first treated with hypoxic conditions for 18 h and thereafter re-oxygenated and grown under aerobic conditions for either 30 h for T-47D-cells (b), 24 h for NH1K 3025-cells (d) or 20 h for T-47DHU-res-cells (f). Cells were analysed as described in Fig. 2 and the histograms were extracted from BrdUrd-positive windows of 2-parametric DNA versus BrdUrd-histograms as demonstrated in Fig. 2.

treatment (Fig. 2a and c) were still present in S-phase, both immediately following the hypoxic treatment (Fig. 2d and f) and after re-oxygenation and exposure to aerobic conditions for 30 h (Fig. 2g and i). There was, however, a difference between panels (i) and (f) as in panel (i) there was a minor tendency of a shift towards higher DNA-content per cell than in panel (f). Thus, there may have been a small increase in the amount of DNA per cell for cells in early S over the 30 h of aerobic conditions following re-oxygenation, indicating that S-phase arrest in these cells was not completely irreversible. In contrast, however, BrdUrd-non-labelled cells (BrdUrd⁻), i.e. cells that were in G₁-phase at the start of the hypoxic treatment (Fig. 2a and b), clearly had resumed cell-cycle progression after re-oxygenation (Fig. 2g and h). This is observed from the significant S-phase fraction of the population represented in panel (h). From panels (d) and (e) it is seen that the BrdUrd⁺ subpopulation had no G₂-fraction following the 18 h of hypoxia. We have interpreted this as a confirmation of our earlier observations, that the T-47D cells initially in G₂-phase before the treatment are able to progress through the G₂-phase and divide during the 18-h treatment period. Thus, arrest induced by prolonged exposure to extreme hypoxia in T-47D cells rendered hypoxic while in S-phase is protracted up to at least 30 h following re-oxygenation, while the arrest in G₁ is quickly reversed after re-oxygenation.

To investigate whether the reversibility of hypoxia-induced arrest in S-phase corresponds with the functionality of pRb, non-pRb-functional NH1K 3025 cells were pulse-labelled with BrdUrd and exposed to extremely hypoxic conditions for 18 h, followed by re-oxygenation and further growth under aerobic conditions for 30 h (Fig. 3c and d). By comparing panels (c) and

Figure 4. Two-parametric DNA vs. BrdUrd histograms for T-47D cells (a), NHIK 3025 cells (b) and T-47DHU-res cells (c). Exponentially growing cells were labelled with BrdUrd for 30 min under aerobic conditions before an 18-h exposure to extremely hypoxic conditions (< 4 p.p.m. O_2) followed by re-oxygenation and exposure to aerobic conditions for 20 h. The cells were analysed as in Fig. 2.

(d) it is seen that BrdUrd-labelled cells increased their DNA-content markedly during the 30 h under aerobic conditions following hypoxia. This experiment, thus, shows that BrdUrd-labelled NHIK 3025 cells exposed to extremely hypoxic conditions while in S-phase are able to resume cell cycle progression within a few hours after re-oxygenation (Fig. 3d). We have previously reported, however, that very few of these cells are able to complete mitosis (Åmellem *et al.* 1996).

The effect of having an increased level of ribonucleotide reductase upon the pRb-mediated regulation in S-phase under extreme hypoxia is demonstrated in Fig. 3(e and f). This was examined using T-47DHU-res cells and providing the same treatment to these as was given to wild-type T-47D-cells and to the NHIK 3025-cells. A comparison between panels (e) and (f) of Fig. 3 shows that in the T-47DHU-res cells, exposed for 18 h to extreme hypoxia followed by re-oxygenation, there is a marked increase in the DNA-content per BrdUrd⁺ cell and this is clearly more significant than the very small increase seen in wild type T-47D-cells (Fig. 3a and b). Furthermore, the quantity of DNA per cell in T-47DHU-res-cells seems to continue above the level representing G_2 -amount of DNA, and in Fig. 3(f) the highest levels of DNA per cell are seen to exceed the scale of the DNA-axis.

Endoreduplication of DNA in cells after re-oxygenation

Endoreduplication of DNA has been observed in a fraction of both NHIK 3025-cells and T-47DHU-res-cells upon re-oxygenation after treatment with extreme hypoxia (Fig. 4b and c), while no endoreduplication was observed in wild type T-47D cells (Fig. 4a). Thus, some of the cells of types NHIK 3025 and T-47DHU-res that were not irreversibly arrested in S-phase following re-oxygenation (as shown in Fig. 3c–d and e–f, respectively) seem to have initiated a second round of DNA replication without having completed mitosis.

Clonogenic capabilities of cells after hypoxia

To investigate the clonogenic capability of cells exposed to extreme hypoxia for 18 h while in G_1 , and in S-phase, cells in G_1 and cells in S were, respectively, sorted from untreated cell populations as described in MATERIALS and METHODS. After sorting, the cells were exposed to hypoxia for 18 h followed by re-oxygenation and thereafter incubation in a CO_2 -incubator. After 2–3 weeks of incubation the cells were fixed and colonies counted. Table 1 shows the

Table 1. Fraction of cells able to form colonies after 20-h exposure to hypoxia. Cells having, respectively, G₁- and S-phase DNA-content were sorted prior to the hypoxic treatment as described in MATERIALS AND METHODS. Mean $\pm$ SE are provided. The clonogenic assay is standardized with respect to the plating efficiency of sorted aerobic cells. *t*-test was performed to determine significance

Cell-cycle fraction	Surviving fraction after 20-h hypoxia		
	G ₁ -phase	S-phase	Test G ₁ - versus S-phase
T-47D	0.56 $\pm$ 0.05	0.13 $\pm$ 0.01	<i>P</i> < 0.01
T-47DHU-res	0.56 $\pm$ 0.03	0.17 $\pm$ 0.04	<i>P</i> < 0.01
NHIK 3025	0.08 $\pm$ 0.03	0.03 $\pm$ 0.01	<i>P</i> $\approx$ 0.01
Test T-47D versus T-47DHU-res	<i>P</i> > 0.05	<i>P</i> > 0.05	
Test T-47D versus NHIK 3025	<i>P</i> < 0.01	<i>P</i> < 0.01	

fraction of seeded cells able to form a colony, for all cell types, after treatment with hypoxia. In all three cases, the colony-forming fraction is significantly lower for cells exposed to hypoxia while in S-phase than for cells exposed to hypoxia while in G₁-phase. Irrespective of cell-cycle phase, the colony-forming fraction of NHIK 3025 cells was significantly lower than that of T-47D- and T-47DHU cells. There is no significant difference in colony-forming ability between T-47D and T-47DHU-res cells. The clonogenic assay was standardized with respect to the plating efficiency of sorted aerobic cells.

To test whether the cells that had endoreduplicated their genome after exposure to hypoxia for 18 h were clonogenic, cells that displayed higher than G₂ DNA content 20 h after re-oxygenation were sorted from hypoxia-treated populations by use of a flow cytometer, as described in MATERIALS AND METHODS. Four thousand cells had been incubated per 25 cm² flask for colony formation, and colony numbers were counted after 2–3 weeks of incubation. Cells of both types (NHIK 3025 and T-47DHU-res cells) showed a low colony-count. Of the seeded cells only 0.05 $\pm$ 0.04 and 0.3 $\pm$ 1% formed a macroscopic colony for NHIK 3025 and T-47DHU-cells, respectively.

For T-47DHU-cells, we also checked whether the colony-forming cells maintained their abnormally high DNA-content over time. This was performed by isolating one colony of the endoreduplicated T-47DHU-res cells and plating these in a new flask where they were allowed to grow for a further 6 weeks before the cells were fixed and prepared for DNA-measurement in the flow cytometer as described above. In Fig. 5, DNA-histograms are shown for wild-type T-47DHU-res cells (a) as well as for cells descending from the colony formed by sorted cells (b). As an internal control, the two populations were also mixed before flow-cytometry in order to ascertain that the relative amounts of DNA were not influenced by stainability nor amplification differences (c). The data show that cells descendant from the colony were clearly of the type having reduplicated DNA and, furthermore, that the doubling of DNA-content was kept stable over weeks.

Classification of the important R2 subunit of RNR in T-47DHU-res cells

There are two different types of RNR enzyme in mammalian cells. Both are tetrameres consisting of a dimer of the R1 subunit, plus a dimer of a further subunit which differs depending on the type of RNR (Kolberg *et al.* 2004). The RNR acting in normal S-phase uses the subunit denoted R2 while RNR acting following DNA damage uses the subunit denoted p53R2 (Engström *et al.* 1985; Tanaka *et al.* 2000). Whether it was the R2 subunit or the p53R2 subunit

Figure 5. DNA-histograms of aerobic untreated T-47DHU-res cells (a), endoreduplicated T-47DHU-res cells (b) and a mixed population of untreated/endoreduplicated T-47DHU-res-cells (c). The endoreduplicated T-47DHU-res cells were obtained by first exposing T-47DHU-res cells to hypoxia for 18 h, re-oxygenation and growth under aerobic conditions for 24 h before endoreduplicated cells were sorted and grown aerobically. DNA was stained with Hoechst 33258 and measured by flow cytometry as described in MATERIALS AND METHODS. Data were analysed with the ModFit (Verity Software) program.

which was increased in the T-47DHU-res cells compared with the T-47D cells has been clarified using western blotting (Fig. 6). The western blot indicates that the level of the p53R2 subunit is almost identical in T-47D and T-47DHU-res cells while it is the level of the R2 subunit which is markedly increased in the T-47DHU-res cells.

DISCUSSION

In the present study, three types of cells were exposed to extremely hypoxic conditions (i.e. < 4 p.p.m. O_2). In living tissues, a low level of oxygenation such as this might arise due to either blockage of blood vessels or lack of vascularization, in, for example, solid tumour areas. At this oxygen level, cell respiration is severely hampered. We measured the nuclear binding of pRb in relation to the cell cycle both during hypoxic treatment and after re-oxygenation. The findings indicate that pRb-activation prevents S-phase cells from continuing undue cell-cycle progression

Figure 6. Western blot of p53R2 and R2 subunits of RNR. Aerobically grown T-47D and T-47DHU-res cells were harvested and analysed as described in MATERIALS and METHODS. Samples contained equal quantities of cells.

following re-oxygenation. This is well in line with our previous findings (Graff *et al.* 2002) which have shown pRb to be activated in S also under conditions of moderate hypoxia (i.e. 1300 p.p.m. O_2) where cell growth is inhibited while the oxygen concentration is still high enough so that cell respiration is unlimited by oxygen supply. Under those conditions, pRb-activation has been found to strengthen cell-cycle inhibition induced by hypoxia both during hypoxia and after re-oxygenation. Our present results confirm previous findings that pRB may prevent cells from endoreduplicating their DNA following cell-cycle arrest (Stokke *et al.* 1997; Niculescu *et al.* 1998), but in our case prevention has taken place following an arrest in S-phase while Niculescu and colleagues studied a p21-induced G_2 -arrest. Surprisingly, however, is our observation that this protective mechanism of pRb seems to be overrun in cells having an increased level of RNR. The present data also indicate that pRb-activation may have a protective function with respect to cell survival, and that this function is not affected by an increased level of RNR. As the cell types used differ in many respects, there is a possibility that variations other than the ones investigated could play a role, but the data still indicate that pRb and RNR are important for regulation during and after exposure to hypoxia.

S-phase arrest during hypoxia is independent of pRb

Cells of all three lines were arrested throughout S-phase upon exposure to extremely hypoxic conditions. As reported earlier, the almost immediate halt in DNA synthesis and replication observed are due to specific inhibition of oxygen-dependent enzymes (Åmellem *et al.* 1994; Graff *et al.* 2002), and inhibition of replicon initiation (Probst *et al.* 1988; Probst *et al.* 1999). Two observations support the notion that these mechanisms of arrest are pRb-independent. First, S-phase arrest is induced equally effectively in non-pRb-functional NHIK 3025 cells as in pRb-functional T-47D cells. Secondly, dephosphorylation and nuclear binding of pRb is a slow process requiring more than 4-h treatment with extremely hypoxic conditions in order to become effective (Åmellem *et al.* 1996). Thus, pRb is neither sufficient for, nor even required for induction of the immediate arrest in S-phase under extremely hypoxic conditions, but seems to be important for regulating re-entry into the cell cycle following re-oxygenation. One candidate for this immediate halt in the DNA synthesis is RNR. RNR is deactivated under extremely hypoxic conditions as there is no signal from the RNR tyrosyl radical in cells grown under such conditions as measured by EPR and performed previously (Graff *et al.* 2002); also the T-47DHU cells, in which the tyrosyl radical can be easily observed did not show any radical EPR signal (data not shown).

Regulation of pRb under extremely hypoxic conditions and following re-oxygenation

pRb is similarly dephosphorylated and re-bound in the nucleus of S-phase cells during prolonged extreme hypoxia in wild-type T-47D cells as in cells of the HU-resistant subtype T-47DHU-res

(Fig. 1). In both cell types, more than 90% of the cell nuclei in S and G₂-phases are found within the pRb⁺ region after 18-h exposure to extremely hypoxic conditions (Fig. 1b and g). This is consistent with our earlier findings that an increased level of ribonucleotide reductase (eight times more radical from induced RNR R2 subunit in T-47DHU-res cells than in T-47D cells) seems not to change pRb binding to the nucleus under hypoxic conditions, although it may nonetheless change the cells' response to hypoxia (Graff *et al.* 2002).

In both cell types, this nuclear pRb binding is very protracted even following re-oxygenation. Recruitment of cells into S- and G₂ phases during the first 6 h following re-oxygenation, as indicated in Fig. 1(c and h), comes from the pool of pRb-negative G₁-cells (i.e. G₁-cells having little nuclear-bound pRb). This is shown in Fig. 2(f and i) where none of the T-47D cells originally in S-phase by the onset of hypoxia (i.e. BrdUrd⁺) are seen to have entered G₂-phase following 18 h of hypoxia, and not even 30 h following re-oxygenation (Fig. 3a and b). Thus, the elevated level of RNR in the T-47DHU-res cells as compared with the wild-type cells seems not to have influenced the nuclear binding of pRb, at least up to 6 h following re-oxygenation. Still, the elevated level of RNR must have affected the function of pRb in the long run as T-47DHU-res cells arrested in S-phase during extremely hypoxic conditions, in contrast to wild-type T-47D-cells, are able to resume and more or less complete DNA-synthesis during a period of only 20 h following re-oxygenation (as seen by comparison of Fig. 3e and f). It is, thus, probable that re-start of DNA-synthesis may take place even in some T-47DHU-res cells where pRb is still actively bound in cell nuclei (Figs 1g and 3f).

Furthermore, the data in Fig. 4(c) show that a considerable fraction of the T-47DHU-res cells increases their quantity of DNA above the G₂-level, indicating that these cells may have started a new round of DNA replication without completing cell division. As is seen from Fig. 4(c) this endoreduplication takes place both in cells that were originally in S-phase at the onset of hypoxia and in cells out of S and arrested in G₁ during hypoxia. Little or no indication of such DNA-over-replication is seen in the wild-type T-47D cells (Fig. 4a).

In NHIK 3025 cells (Fig. 3c and d), known to be defective with respect to pRb-function due to the presence of HPV18 E7 oncoprotein (Fig. 1d and e), both resumption of DNA synthesis in cells arrested in S-phase during hypoxia and endoreduplication of DNA in some cells originating from S as well as from G₁ is seen following re-oxygenation. As T-47DHU-res cells thus, similar to the wild-type T-47D cells, have pRb re-bound to the nucleus as induced by hypoxia, while still behaving more like the pRb-defective NHIK 3025-cells following re-oxygenation, our conclusion is that the elevated level of RNR in the T-47DHU-res cells may have overruled the pRb-inflicted restriction of cell-cycle progression following re-oxygenation. Thereby, the possibility is that abnormally high levels of RNR may have an oncogenic function under strictly hypoxic conditions. First, there may be a selection of cells having increased RNR as these have an improved ability for cell-cycling and production of new cells following re-oxygenation. Secondly, some of the selected cells may have an increased genomic instability through an abnormal elevation of the DNA-content of the cycling cells.

pRb regulates re-entry into the cell cycle after re-oxygenation and prevents endoreduplication of DNA

S-phase arrest induced by hypoxia in cells rendered hypoxic while in S-phase seems to be very protracted in wild-type T47D-cells (Fig. 3). Thus, even 30 h after re-oxygenation, these cells had not synthesized any significant amount of DNA. The small increase observed may in fact be as a result of repair processes activated after re-oxygenation as we have previously shown that these cells incorporate some BrdUrd over the first 30 min following re-oxygenation, but still do not increase their quantity of DNA for the next 20 h (Åmellem *et al.* 1996). In contrast, both the

NHIK 3025 cells and the T-47DHU-res cells that were in S-phase at the onset of hypoxia were able to reinitiate S-phase progression after re-oxygenation.

As indicated in Fig. 4, both NHIK 3025-cells and T-47DHU-res-cells also appear to over-replicate DNA following re-oxygenation. Although this is seen in only a small subfraction of cells from each of the two cell types, there is a clear difference between these two cell types and the wild-type T-47D-cells for which no such over-replication has been detected. Because hypoxia-arrested S-phase cells of the wild type T-47D line are unable to even resume DNA-synthesis following re-oxygenation, it is not surprising that these cells do not over-replicate DNA following re-oxygenation. We believe that nuclear binding of pRb is the factor responsible for the prolonged inhibition in these cells. It is, however, worth noticing that the RNR-rich cells of the T-47DHU-res line, having seemingly equally nuclear binding of pRb as the wild-type T-47D-cells, both resume DNA-synthesis and over-replicate DNA following re-oxygenation in much the same way as the pRb-incompetent NHIK 3025-cells. Taken together, the present results indicate that an increased level of RNR might somehow counteract the pRb-induced regulation of DNA-synthesis during and following extreme hypoxia. The observed endoreduplication is probably not induced by addition of the DNA damaging agents BrdUrd or Hoechst 33342, as endoreduplication is observed both in cells which have not been exposed to Hoechst 33342 (Fig. 4) and in cells not exposed to BrdUrd (Fig. 5). Also, endoreduplication is not observed in the T-47D cells exposed to BrdUrd (Fig. 4a). We therefore believe that hypoxia is the inducer of the observed endoreduplication.

Hypoxia-induced endoreduplication of DNA has also been observed in murine tumour cells, and correlates with an enhanced metastatic potential of such cells (Young *et al.* 1988). This is well in line with conclusions that can be drawn from the present data. Lost or reduced pRb function in tumour cells, for example by infection with the HPV 18 virus as in the NHIK 3025-cells, or by cells having an elevated level of ribonucleotide reductase as in the T-47DHU-res-cells, may lead to over-replication of DNA and consequently increased genomic instability in a hypoxic microenvironment. The capacity to irreversibly block replication of DNA damaged during hypoxia may thus be an important function of pRb and loss of this regulation may particularly increase malignant selection under hypoxic conditions. This view is further supported by the finding that DNA damage induced by cisplatin results in DNA endoreduplication and cell death in pRb^{-/-} MEFs, while pRb^{+/+} MEFs are permanently arrested (in S and other phases) with pRb in its underphosphorylated state (Knudsen *et al.* 2000).

pRb may increase cell survival following extreme hypoxia

Over-replication of DNA in cells might lead to changed cellular characteristics and might be lethal to an organism, if these cells were able to multiply. To investigate the importance of pRb for survival after exposure to extremely hypoxic conditions, the surviving fraction was measured following 20-h treatment with extremely hypoxic conditions for cells of all three types (Table 1). The results show that NHIK 3025 cells are most sensitive to hypoxia and that less than 10% of these cells are able to form colonies following this treatment. For NHIK 3025-cells selected with S-phase DNA content the surviving fraction was found to be only 3%. This supports the earlier finding that almost all of the S-phase NHIK 3025 cells are inactivated following protracted hypoxia (Åmellem & Pettersen 1991) and that those able to continue DNA synthesis after hypoxia are subsequently arrested in mitosis (Åmellem *et al.* 1996). The logical consequence of these findings is that pRb might not only be involved in protecting the organism from lethal damage acquired in S-phase by protracted arrest of the cells in S-phase, but may also protect the individual cell from the damaging effects of extreme hypoxia, thus increasing the cell surviving fraction. The same effect of pRb dephosphorylation is also observed for T-47D cells when

Table 2. Cell cycle distribution of wild-type T-47DHU-res cells and T-47DHU-res cells with endoreduplicated DNA (Fig. 5a and b, respectively). The cell cycle distribution was analysed using ModFit (Verity Software)

	G ₁ -phase	S-phase	G ₂ -phase
T-47DHU-res	52%	25%	23%
Endoreduplicated T-47DHU-res	58%	24%	24%

exposed to low dose-rate irradiation, where data have indicated that the presence of functional pRb might increase cell survival (Furre *et al.* 2003). Thus, pRb regulation may have a general function both as a cell-cycle regulator and as a stress-protective factor in mammalian cells.

It has earlier been reported that the ribonucleotide reductase subunit R2 might function together with other oncogenes, and increase the malignant potential of the cancer cells (Fan *et al.* 1996, 1998). We have previously proposed that this might be due to the growth advantage an increased level of ribonucleotide reductase gives cells in a moderately hypoxic microenvironment (Graff *et al.* 2002). Table 1 shows that T-47DHU-res cells and T-47D cells exhibit the same surviving fraction following treatment with extreme hypoxia and, parallel to what is observed for NHIK 3025 cells, the surviving fraction of cells in G₁-phase is much higher than for cells in S-phase. The finding that T-47DHU-res cells are equally resistant as wild-type T-47D cells shows that the increased level of RNR in the T-47DHU-res cells does not affect cell survival after treatment with extreme hypoxia even though the increased level of RNR induces endoreduplication of DNA in many of the T-47DHU-res cells.

An important question is whether or not a few of the T-47DHU-res cells, having abnormally high DNA-content, are clonogenic. We performed flow cytometric sorting of T-47DHU-res cells with more than G₂ DNA content, seeded the cells for colony formation and found that less than 3% of the sorted T-47DHU-res cells were able to form colonies (data not shown). Thus, the colony-forming ability of the subpopulation having abnormally high DNA-content is certainly low. Still, from the data in Fig. 5 it is evident that some of the surviving cells have a doubled DNA-content and these cells have maintained their elevated level of DNA for several weeks. The cell cycle distribution of the cells in Fig. 5(a and b) is also similar (Table 2). However, a fraction of cells (approximately 7%) with normal G₁ DNA content was observed, indicating that this cell line is not stable (Fig. 5b). This could also result in an overestimation of the endoreduplicated G₁-peak as this might also consist of some normal G₂ cells.

Oncogenic function of elevated RNR

The finding that an elevated level of RNR in the T-47DHU-res cells seems to overrule the pRb-inflicted restriction of cell-cycle progression following re-oxygenation is an indication of a possible oncogenic effect. Whether it was the normal R2 subunit of RNR or the p53R2 subunit which was important for the properties of T-47DHU-res cells, was resolved using western blotting. We have previously investigated the cells using EPR (Graff *et al.* 2002), but with that method it is impossible to differentiate between the normal R2 subunit and the p53R2 subunit of RNR. However, the current western blot has now shown a greatly increased level of R2 in the T-47DHU-res cells compared with the T-47D cells, while the level of p53R2 was close to equal in the two cell lines (Fig. 6).

But is RNR an oncogene? As was earlier pointed out by Baserga (1999) 'almost anything that is overexpressed can lead to cell transformation, including glycolytic enzymes'. The oncogenic effect in this case is a result of RNR overexpression in the T-47DHU-res cells as a consequence

of their adaptation to the highly toxic effect of hydroxyurea. Nevertheless, this finding may nourish some reflections concerning growth regulation under hypoxic conditions. If tissue hypoxia contributes to cancer development in this way, it must be vital for the cells to deactivate RNR immediately following reduced oxygenation and it is easy to understand why RNR-activation by molecular oxygen is such a highly conserved mechanism. Oxygen is needed for the generation of activity dependent tyrosyl radical and the di-iron-oxygen cluster from the apo RNR R2, Fe(II) and oxygen (Thelander & Gräslund 1993; Andersson & Gräslund 1995; Sjöberg 1997). The moment a cell senses severe lack of molecular oxygen, RNR is possibly deactivated. This regulation pathway is not the oxygen-sensing mechanism relating to the Hif-pathway, but is by a mechanism so simple that there is no need to wait for synthesis of new gene products or degradation of existing ones, and there is no need to wait for metabolic processes or even kinase or phosphatase activity:

In mammalian systems, this radical is rapidly destroyed and a continuous presence of oxygen is needed to maintain activity (Thelander *et al.* 1983). By reducing the amount of molecular oxygen available, less RNR will be activated and DNA synthesis might be turned off due to lack of deoxynucleotides. The problem is, however, that increased quantities of RNR in T-47DHU-res cells will shift the equation to the right, activating some RNR at a reduced oxygen level. Some of the RNR-function is thereby restored, although conditions are not in the favour of DNA-synthesis. Even in pRB-functional cells this may become a problem as the protection offered by pRb-activation in S-phase under hypoxic conditions is a slow process, taking several hours to become operational. Thus, in presence of elevated RNR, some DNA synthesis may be commence under relatively severe hypoxia. Thereby, the possibility is that abnormally high levels of RNR may have an oncogenic function under such hypoxic conditions, as there may be a selection of cells having increased RNR as these have an improved ability for cell-cycling and production of new cells following re-oxygenation.

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