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CELL CYCLE DISTURBANCES INDUCED BY RADIATION AND OTHER CELLULAR STRESSES

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Table of contents:

1. Introduction 3

2. Cell cycle delays after irradiation and the role of the p53 protein

2.1 G_1-block 5

2.2 S-phase delay 8

2.3 G_2-block 11

3. Quiescent S-phase cells *in vitro* induced by different physical and chemical factors

3.1 Induction of quiescent S-phase cells by radiation and/or hyperthermia 13

3.2 Induction of quiescent S-phase cells by chemicals 15

4. Occurrence of quiescent S-phase cells under extreme physiological conditions

4.1 Low pH, hypoxia, and serum deprivation *in vitro* 17

4.2 Tumour tissue *in vivo* 22

5. Prospects for future fundamental and applied research 25

Resumé 28

Resumé (český) 30

Literature 32

Publications that form the basis of this dissertation 37

1. Introduction

All studies presented are based on the method of flow cytometry. It facilitates the identification and counting of thousands of cells within a few seconds. After appropriate staining or labelling, cells in a suspension are passed through a narrow light beam. Light scatter and/or fluorescent signals generated by each single cell are measured and then processed for further analysis.

The first measurements of DNA content of individual cells which were precise enough to distinguish between different phases of the cell cycle were made around 1970 (Dittrich und Göhde, 1969). A further improvement of cell cycle analysis was made possible with the development of monoclonal antibodies (Köhler and Milstein, 1975). Particularly important was the introduction of a method which allowed the simultaneous measurement of DNA content and incorporated DNA precursors (Dolbeare et al., 1983). Cells were pulse labelled with bromodeoxyuridine (BrdU), an analogue of thymidine, and a monoclonal antibody against this compound – conjugated with a fluorescent dye such as FITC - was used as a probe for its incorporation into the DNA. Compounds such as propidium iodide have continued to serve as a probe for total DNA content.

The cell cycle distribution could now be assessed on the basis of a characteristic cell function carried out in S-phase, namely DNA synthesis. What was even more important, however, was the fact that this made it possible to distinguish between cells which were actively progressing through the cell cycle and those that just “looked as if” because they had a DNA content intermediate between G_1 and G_2M.

Early indications of the existence of quiescent S-phase cells had been obtained with cell cultures kept under extreme physiological conditions such as hypoxia and low pH, which are typical of solid tumours, especially at an advanced stage. Hypoxia *in vitro* led to a strong reduction of the BrdU labelling index without any concurrent change in the DNA histogram. The authors’ interpretation was that not all S-phase cells were actively synthesizing DNA (Shrieve et al., 1983). Similarly, low pH *in vitro* completely inhibited growth of human tumour cells, but had practically no influence on the cell cycle distribution. The authors of this study concluded that “cells must have been unspecifically arrested in all cell cycle phases” (Taylor and Hodson, 1984).

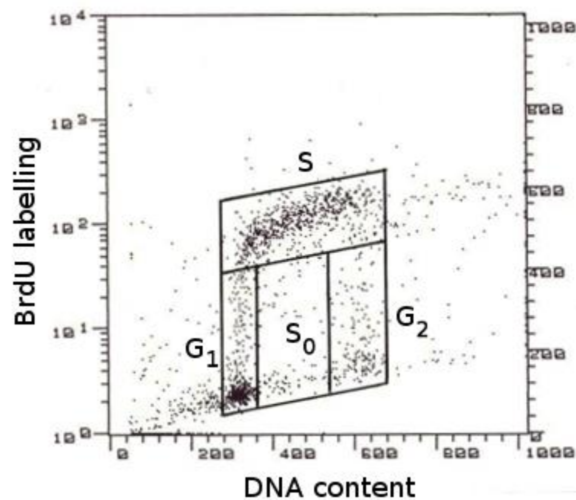

Fig. 1: Example of a two-parameter scattergram.

For each individual cell, the red fluorescence signal from propidium iodine is plotted on the x-axis and the green fluorescence signal from an FITC-conjugated antibody against BrdU is plotted on the y-axis. Cells are assigned to different cell cycle compartment, S_0 signifying cells which have a DNA content typical of the S-phase, but have not incorporated BrdU.

At the time, two-parameter flow cytometry after BrdU labelling was just coming up, so the evidence remained indirect. A little later, however, the method was employed in animal as well as human tumours and evidence of the existence of unlabelled S-phase cells was obtained in many cases (Wilson et al., 1985; Riccardi et al., 1988).

The results were criticized, because the BrdU which had been injected intravenously might not have reached all cells if vascularization was inefficient (Wilson et al. 1985). But the fact that these observations were in line with the above mentioned evidence *in vitro* made the case stronger for the possibility that quiescence occurs outside G_0.

Already before the introduction of the BrdU method, there had been some direct evidence for the existence of such cells. For instance, on the basis of flow cytometric measurements using acridine orange, a compound whose staining properties depend not only on the total DNA content of a cell, but also on its condensation state, it was concluded that while lymphocytes of healthy donors rested only in G_0, those of leukaemia patients went into a similar state of quiescence in G_1, S or G_2 as well (Darzynkiewicz et al., 1979). In agreement with these findings, others reported that when cells were labelled with radioactive ^{3}H-thymidine, and the DNA content of labelled and unlabelled cells was then determined, not all cells with an S-

phase DNA content had incorporated the ^{3}H-thymidine (Allison et al., 1983; Drewinko et al., 1984). Even in cell cultures growing under optimal conditions, a few percent of the S-phase cells would be unlabelled, but the fraction rose significantly after radiation and/or hyperthermia treatment (Streffer et al., 1980; Streffer et al., 1983).

This was the starting point of the work presented here. The hypothesis that quiescent S-phase cells could be induced by exposure to various physical and chemical agents was to be tested with the help of flow cytometry after BrdU labelling. At the same time, we decided to look more closely at the occurrence of quiescent S-phase cells under extreme physiological conditions such as low pH and hypoxia *in vitro* as well as *in vivo*, because these conditions are related to modifications in the radiation response of cells. It also soon became obvious that there was a need to conduct dynamic studies of the movement of cells through the cell cycle instead of just taking snapshots of the cell cycle distribution at particular points in time. The following presentation of our data will begin with this last-mentioned aspect.

2. Cell cycle delays after irradiation and the role of the p53 protein

2.1 G_1-block

When the investigations described here were started, it had just been reported that the radiation induced G_1-block depended on the expression of a p53 wild-type protein (Kastan et al., 1991; Kuerbitz et al., 1992). In those previous studies, exponentially growing cell cultures had been used and it was unknown what the effect on serum starved and subsequently stimulated cell cultures would be. Also, the time course of the progression from G_1- into S-phase, or as in our case, from G_0- into G_1- into S-phase had not been studied in detail.

F. Zölzer, S. Hillebrandt, and C. Streffer, Radiation induced G_1-block and p53 status in six human cell lines. Radiother. Oncol. 37 (1995) 20-28

In this study, four human tumour cell lines were used: two melanoma cell lines (MeWo, Be11) and two squamous carcinoma cell lines (4197, 4451). We also included two fibroblast strains in order to compare with untransformed cells. All cell types were first analysed as to

their p53 status. Expression of p53 was investigated with the help of a microscopic immunofluorescence assay and other methods, and the state of the p53 gene was further analysed with the help of temperature gradient gel electrophoresis (TGGE). These experiments showed that of the six cell types mentioned, four were p53 wild types, whereas two were mutants, having an altered base sequence in exon 7 of the p53 gene.

In order to follow the movement of cells from G_0- into G_1- into S-phase we took the approach suggested by Nagasawa et al., 1984. Cells were irradiated after 6 days of serum starvation. Fresh medium was added and the cells were labelled with BrdU for 30 min at different times afterwards. As expected, only a small percentage of cells were labelled immediately before release from growth inhibition, depending on the cell line used. After some time, however, typically 8 hours for otherwise untreated cell cultures, the labelling index rose, indicating entry of cells into the S-phase. With cultures irradiated shortly before stimulation the entry into S was delayed, but only in those cell types that had a functional p53 protein. The delay time was roughly proportional to the X-ray dose. The two p53 mutant cell lines either showed no change in the $G_{0/1}$-S progression, or even a slight acceleration. Whereas all tumour cell lines, irrespective of a radiation effect on the time of entry into S-phase finally reached the same maximum labelling index, the two fibroblast strains showed a clear reduction in this parameter, suggesting a permanent block of some cells in G_0 or G_1.

Apart from confirming the finding about the importance of p53 for the radiation-induced G_1-block, our experiments showed that the checkpoint can be activated not only immediately before the G_1/S transition, but several hours earlier, when the cells are still in the G_0-state.

We also found that within each of the two pairs of tumour cell lines, the p53 wild type was more radiation resistant, whereas the mutant was sensitive. This was in accordance with expectations, because the G_1-block should provide more time for DNA repair and therefore increase survival. In quite a few other studies, however, p53 wild types were found to be more radiation sensitive as compared to mutants, which shows that matters are more complex than initially thought and the ability undergo a G_1-block is not the only decisive factor for cellular survival (Bristow et al., 1996; El-Deiry, 2003).

F. Werner, F. Zölzer, and C. Streffer, p53 levels, cell cycle kinetics and radiosensitivity in two SV40 transformed Wi38VA13 fibroblast strains. Strahlenther. Onkol. 177 (2001) 662-669

As a corollary to the earlier study involving a number of genetically unrelated cell lines, we later used two SV40 transformed fibroblast strains, one of which was derived from the other. The new strain had a strongly reduced expression of SV40 large T antigen, a protein known to complex with p53. Two commercially available antibodies against the latter were used to assess its detectability in these two cell types. A detailed analysis led to the conclusion that both antibodies were able to bind to free p53, but that they had different affinities to p53 complexed with the SV40 large T-antigen.

Again using the approach of Nagasaki et al. to follow the movement of from G_0- into G_1- into S-phase after irradiation, we observed a clear delay in the (new) strain with low p53 expression, but no significant change in the number of S-phase cells and no cell cycle delay in the (original) strain with high p53 expression. That was surprising at first sight, but could be explained by assuming that the complex formation of p53 with the SV40 large T antigen increased its concentration (presumably by affecting its life time), but at the same time affected its functionality so that it did not perform its cell cycle checkpoint function in spite of it being present in a high concentration.

Interestingly, we observed the same order of sensitivities in this case as described above: the strain with a functional p53 was more resistant than the one with a reduced functionality. Because otherwise the two strains could be assumed to be completely identical, we concluded that the dominant factor here was indeed the ability to undergo a G_1-block and that its influence on cell survival was as initially expected.

F. Zölzer and C. Streffer, Relative Biological Effectiveness of 6 MeV neutrons with respect to cell inactivation and G_1 block. Radiat. Res. 169 (2008) 207-213

A few years later we had the opportunity to expose cultured cells to neutrons produced at the Essen cyclotron facility. These had a mean energy of about 6 MeV and therefore a relatively high LET (linear energy transfer) of 10 – 100 keV/µm. In survival experiments, their RBE (relative biological effectiveness) was shown to be smaller for sensitive cells and higher for resistant ones.

We analysed the G_1-block in four cell types, all of them p53 functional (Be11, 4197 and one of the fibroblast strains as in the first study above, as well as human glioma EA14). Similar to our first study, we observed a G_1-delay, which means a radiation-induced shift in the entry of cells into the S-phase, and a G_1-arrest, which is reflected in the reduction of the maximum percentage of cells in S-phase reached after radiation. Both phenomena were induced by neutrons more effectively than by X-rays, but there were interesting differences. While the RBE for the G_1-delay was the same for all cell types used, the RBE for the G_1-arrest closely resembled that for loss of colony forming ability, i.e. it correlated with radiation sensitivity.

These findings were not only new, because nobody had at the time (and to date) looked at neutron-induced disturbances of the G_1-phase, but they also provided some insight into how cell cycle delays are produced. We reasoned that because X-rays and neutrons produce similar numbers of strand-breaks per dose (Prise et al., 1998), a RBE of higher than 1 must be due to different processing of X-ray induced and neutron induced strand-breaks. From our observations we concluded that the induction of a G_1-delay is an early event independent of the repair capacity, because the RBE is similar in all cell types investigated. The G_1-arrest, however, is more closely related to the later events leading to cell inactivation, where strand-break repair does play a major role, influencing X-ray sensitivity more strongly than sensitivity to neutrons because of a lower repairability of lesions induced at higher LET.

2.2 S-phase delay

F. Zölzer, P. Uma Devi, and C. Streffer, Determination of potential doubling times in human melanoma cell cultures subjected to irradiation and/or hyperthermia by flow cytometry. Radiat. Res. 138 (1994) 451-459

While pulse-labelling cells with BrdU and fixing them for analysis immediately afterwards gives a “snap-shot” picture of the cell cycle distribution at that point in time, it is also possible to use the BrdU labelling technique for an analysis of the movement of cells through S-phase and further on. This is done with a so-called “pulse-chase” protocol, in which cells are labelled with BrdU for a brief time, left to progress through the cell cycle for a number of hours, and only then fixed and analyzed. Such an approach had been used by Begg et al.,

1985, to prognosticate the growth of human tumours, but it had not yet been applied to the analysis of cell populations after irradiation or other cellular stresses. In a series of experiments, we therefore investigated the response of human melanoma cells (p53 mutant Mewo, see next section for other cell lines) to irradiation and/or hyperthermia.

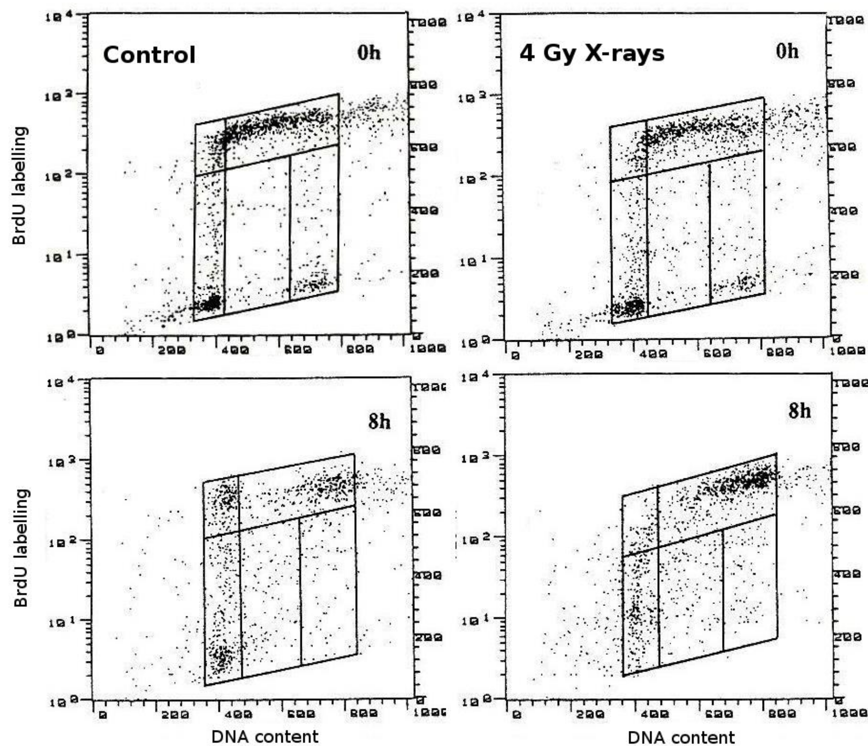

Fig. 2: Scattergrams of human melanoma cells (MeWo) from a "pulse-chase experiment"
Cells were labelled for 30 min immediately after treatment and then incubated for 0 or 8h in BrdU-free medium, after which they were fixed and analysed.
Note how the labelled S-phase cells move to higher DNA contents with time, and 8 h after labelling some of the control cells have already entered the next G_1-phase.

The scattergrams above show the type of results obtained from such experiments. When cells are fixed immediately after BrdU labelling, the labelled cells all have an intermediate DNA content between those in G_1 and G_2. With a later fixation time, the cloud of labelled cells moves to the right showing progress of the labelled cells through the S-phase and finally labelled cells begin to appear in the G_1-compartment after having gone through mitoses. The "Relative Movement" defined as

$$\mathrm{RM} = \frac{F_{\mathrm{L}} - F_{\mathrm{G_1}}}{F_{\mathrm{G_2}} - F_{\mathrm{G_1}}}$$

i.e. the position of labelled cells relative to those in G_1 and G_2, is directly proportional to the time after labelling and the slope (up to the point in time where labelled cells start entering the next cell cycle) is equal to the inverse of the duration of S-phase (White and Meistrich, 1986).

This analysis was carried out for unexposed cells and for cells treated with irradiation and/or hyperthermia. It was found that the duration of S-phase increased by a few hours after moderate doses of X-rays, but considerably more after hyperthermia or a combination treatment. The radiation induced delay was still present 48 h after exposure (i.e. when cells were labelled with BrdU at this time and their progress from that point onwards was analysed). The delays induced by hyperthermia alone or in combination with radiation were much reduced 48 h after treatment, but still longer than those induced by irradiation alone.

At the time we were mainly interested in the possibilities of prognosticating the further growth of the treated cell cultures (which turned out to be rather problematic). In the present context, this study is taken as a preparatory exercise for the analysis of S-phase delays in cell lines of different p53 status.

F. Zölzer, T. Mußfeldt, C. Streffer, Differential S-phase progression after irradiation of p53 functional versus non-functional tumour cells. Radiol. Oncol. 48 (2014) 354-360

Many pathways seem to be involved in the regulation of the intra-S-phase checkpoint after exposure to ionizing radiation, but the role of p53 has proven to be rather elusive. We therefore had a closer look at the progression of irradiated cells through S-phase in dependence of p53 functionality, using the "pulse-chase" method already employed with the human melanoma MeWo. The three other tumour cell lines from our first study of the G_1-block were also included (human melanoma Be11 as the p53 wild type counterpart of the mutant MeWo and human squamous carcinoma 4197 and 4451, again a p53 wild type and a mutant). For this study we added a pair of human glioma cell lines, p53 functional EA14 and p53 non-functional U87.

While progression through S-phase in p53 deficient cells was slowed down over at least a few hours, it was halted for just about an hour in the p53 proficient cells and then proceeded without further delay or even at a slightly accelerated pace. Whether the p53 effect is on replicon initiation or on elongation in already initiated replicons is impossible to tell from our

data. A complete halt of replication for 1-2 hours in p53 proficient cell lines would suggest that both are affected, but with up to 100 000 origins of replication in a human cell one would probably not notice if elongation in a few already initiated replicons was continued.

Indeed, experiments in which the direct block of a viral origin by p53 binding was studied after γ-irradiation, it was found that initiation was completely shut down, but elongation continued unabated even though the template must still have been damaged (Zhou and Prives, 2003). On the other hand, an investigation of the different effects of irradiation on DNA synthesis in normal and Li-Fraumeni fibroblasts suggested that while normal fibroblasts shut down only initiation, both initiation and elongation were affected in the p53 deficient cells (Mirzayans et al., 1995), which is in good agreement with our results.

2.3 G_2-Block

F. Zölzer and C. Streffer, G_2-phase delays after irradiation and/or heat treatment as assessed by two-parameter flow cytometry. Radiat. Res. 155 (2001) 50-56

As indicated above, “pulse-chase” experiments provide information about the movement of cells not only through the S-phase, but also through G_2 and M into the next cell cycle. This can be monitored by observing the appearance of labelled cells in the G_1 compartment. Results of such studies had been published (Rice et al., 1986; McNally and Wilson, 1986; Higashikubo et al., 1993), but treatment-induced delays were only determined relative to the control, whereas no information was extracted as to the absolute duration of the G_2M-phase.

A detailed mathematical analysis of the movement of labelled cells through the cell cycle carried out by White and Meistrich (1986) and White et al. (1990), lead to an equation for the fraction of labelled divided cells $f^{ld}(t)$, i.e. precisely those cells that have moved through S, G_2 and M, which can be re-written as

$$\ln [1 - 0.5 \times f^{ld}(t)] = -c\,t + c\,T_{G2M}$$

where c is the growth rate and T_{G2M} is the duration of G_2M. This means that if the parameter $\ln [1 - 0.5 \times f^{ld}(t)]$ is plotted against time after labelling, the result should be a linear relationship with slope -c and intercept c $T_{G2M;}$ back-extrapolation of this parameter to the

time-axis (ln [1 – 0.5 x $f^{ld}(t)$] = 0) indicates the duration of G_2/M (for examples of such plots, see Fig. 8 below). Our results first with the melanoma cell line MeWo confirmed that there was indeed always a time range when the relationship was linear, not only for control cultures, but also for those exposed to radiation or other stresses, and both growth rate and the duration of the G_2M-phase could be determined from regression analysis.

This kind of analysis was carried out after irradiation and/or hyperthermia. It was found that both kinds of treatment induced G_2 delays of similar magnitude, that the disturbances were resolved with a similar time course, and that both were susceptible to caffeine. Particularly significant was the fact that these statements about the G_2M-phase were possible in spite of the fact that the concurrent delays in the S-phase were greatly different, namely 8 - 10 times longer after hyperthermia than after irradiation.

F. Zölzer, G. Jagetia, and C. Streffer, G_2-block after irradiation of cells with different p53 status. Strahlenther. Onkol. 190 (2014) 1075-1079

This initial investigation of the G_2-block was later extended to three of our other cell lines in order to assess the importance of p53 function in this context. Experiments were carried out with the melanoma cell lines MeWo and Be11 and with the squamous carcinoma cell lines 4451 and 4197. Within each of these pairs, as will be remembered, the first line was a p53 mutant, the second a wild type. An initial study of the changes in cell cycle distribution after X-ray exposure seemed to suggest that p53 had indeed a role to play: we found that the maximum accumulation in the G_2-compartment occurred much later in p53 mutants than in wild-types. This was in line with the claim of others (Solberg Landsverk et al., 2011) that p53 is important for the maintenance of the G_2-block at 2 – 10 hour after treatment.

However, further experiments, in which the absolute duration of the G_2-phase was determined from the appearance of labelled cells in the next cell cycle (as just described), strongly suggested that this was not due to a longer block in the G_2-phase itself. All cell lines showed delays of roughly 1 h per Gy. We concluded that looking at the accumulation of cells in the G_2-compartment can be rather misleading when differences between p53 wild-types and p53 mutants are investigated. Such an accumulation may be influenced by a modified progression of cells through earlier cell cycle phases, and does not reflect differences in the G_2-block.

3. Quiescent S-phase cells *in vitro* induced by different physical and chemical agents

3.1 Induction of quiescent S-phase cells after radiation and/or hyperthermia

F. Zölzer, C. Streffer, and T. Pelzer, Induction of quiescent S-phase cells by irradiation and/ or hyperthermia. I. Time and dose dependence. Int. J. Radiat. Biol. 63 (1993) 69-76

F. Zölzer, C. Streffer, and T. Pelzer, Induction of quiescent S-phase cells by irradiation and/ or hyperthermia. II. Correlation with colony forming ability. Int. J. Radiat. Biol. 63 (1993) 77-82

As mentioned in the introduction, the main starting point of the work described here was the finding that unlabelled S-phase cells occurred after radiation and/or hyperthermia treatment (Streffer et al., 1980; Streffer et al., 1983). Those early experiments involved labelling with radioactive ^{3}H-thymidine, and determination of the DNA content of labelled and unlabelled cells with the help of microscope cytometry, which not only made them rather tedious, but also less sensitive, because at best a few hundred cells could be examined. In contrast, flow cytometry allows analysis of thousands of cells within a few seconds, so it was decided to carry out a similar study with this method.

Cells of the melanoma line MeWo were grown in exponential culture, exposed to X-rays and/or hyperthermia, and then left to grow further for up to 6 days. Every day, samples were pulse labelled with BrdU and the cell cycle distribution was determined immediately after. As expected, there were very few unlabelled cells with an S-phase DNA content in untreated cultures, but the fraction increased after treatment, depending on dose and time of incubation. Both with radiation and with hyperthermia a significant increase of the frequency of quiescent S-phase cells was seen with dose, but the two treatments acted clearly additively, so that the dose-effect curves for radiation and for radiation combined with hyperthermia were parallel.

The second part of this study was designed to further elucidate the mechanisms involved. As there could be no direct comparison between doses of radiation and heat, the appearance of unlabelled S-phase cells was compared at similar cell survival levels as determined in the colony formation assay. Generally, of course, the two parameters were closely correlated, but there were also interesting differences. At the same level of survival, for instance, quiescent

cells were much more effectively induced by hyperthermia than by radiation. Our conclusion was that quiescence in S-phase is not just an expression of cell death irrespective of the treatment by which it is caused, but it represents a particular form of interphase cell death.

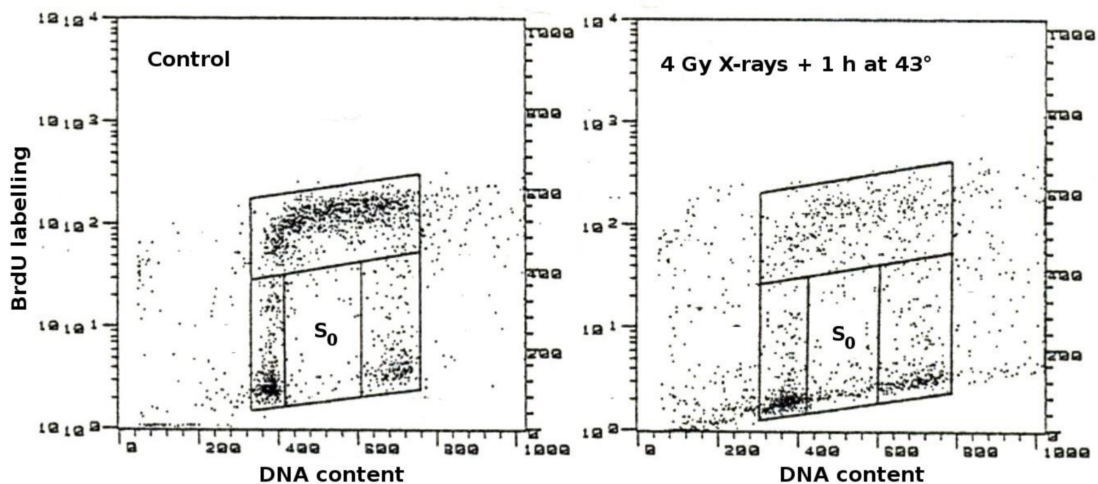

Fig. 3: Scattergrams of human melanoma cells (MeWo) 96 h after pulse labelling. Cells were labelled immediately after sham irradiation or combined X-rays and hyperthermia. Note that the treatment causes an accumulation of unlabelled cells with a DNA content typical of the S-phase, so-called quiescent S-phase cells or S_0-cells.

On a practical note, these observations may be important because they show that cell cycle data derived from one-parameter DNA histograms can be quite misleading, if irradiated or heat treated cell populations are examined. Only a two-parameter measurement can provide information about the actual proliferation state, because a considerable part of the cells seemingly progressing through the S-phase may actually be inactive.

F. Zölzer and C. Streffer, Quiescence in S-phase and G_1 arrest induced by irradiation and/or hyperthermia in six human tumour cell lines of different p53 status. Int. J. Radiat. Biol. 76 (2000) 717-725

The study of quiescent S-phase cells induced by radiation was then extended to other cell lines, namely the above mentioned second melanoma line (Be11), two squamous cell carcinoma lines (4197, 4451) and two glioma lines (EA14, U87). Thus, there were again three pairs of cell lines, each consisting of one p53 functional and one p53 non-functional lines.

These six cell lines were investigated with respect to the appearance of quiescent S-phase cells after treatment. Interestingly, only the lines lacking a functional p53 showed a dose and time dependent increase, while the lines with normal p53 function always showed nothing but background levels of such cells, regardless of dose or time after treatment. These results were interpreted to mean that cells not undergoing a G_1-block have less time available for the repair of DNA damage before entering into the S-phase, which leads to problems during replication and causes some kind of interphase death, as mentioned above. Radiation-induced apoptosis, however, does not seem to play a role here, as it is not unequivocally correlated with the induction of a G_1-block or with p53 status, as least in the set of cell lines used here.

In our discussion of these observations, we came to the conclusion "that the occurrence of quiescent cells in the S-phase compartment is an early manifestation of chromosomal disturbances usually detected many cell generations later." This was in reference to the well-known phenomenon of genomic instability for which there was by then increasing evidence that it was influenced by p53 and the G_1-block. Several authors showed that gene amplification occurred at much higher rates in cells that were unable to halt cell cycle progression before the entry into S-phase (Livingston et al., 1992; Yin et al., 1992; Paulson et al., 1998). While in these studies cells were treated with the uridine biosynthesis inhibitor PALA, it has been suggested that radiation induced genomic instability is also connected with deficiencies in cell cycle regulation (Bartek and Lukas, 2001; Liang et al., 2002). Other authors, however, have argued that it is a functional p53 that is decisive for the prevention of genomic instability, while a functional p21 (which is crucial for the translation of p53 activation into a G_1-block) does not seem be required (Honma, 2005; Zhang et al., 2007). So we may actually be looking here at a direct effect of p53 on DNA repair rather than an indirect effect via checkpoint control.

3.2 Induction of quiescent S-phase cells by chemicals

H. Gong, F. Zölzer, G. von Recklinghausen, J. Rössler, S. Breit, W. Havers, T. Fotsis, and L. Schweigerer, Arginine deiminase inhibits cell proliferation by arresting cell cycle and inducing apoptosis. Biochem. Biophys. Res. Comm. 262 (1999) 10-14

H. Gong, F. Zölzer, G. von Recklinghausen, W. Havers, and L. Schweigerer, Arginen deiminase inhibits proliferation of human leukemia cells more potently than asparaginase by inducing cell cycle arrest and apoptosis. Leukemia 14 (2000) 826-829

So far, the induction of quiescent S-phase cells had been observed only after exposure to physical agents, namely radiation and hyperthermia. We then had an opportunity to investigate the effects of a growth inhibitory protein, arginine deiminase. It is suggested as an alternative to L-asparaginase, a chemotherapy agent which has severe side-effects and moreover appears to be ineffective with some patients. Arginine deiminase has been shown to catalyze the hydrolysis of L-arginine to L-citrulline. Its growth inhibitory effect is therefore generally thought to result from a depletion of arginine. This may indeed be the case, but as shown by our cell cycle studies there must be other factors involved. We used the same BrdU pulse labelling method as described above to compare the growth characteristics of a number of tumour cell lines under the influence of arginine deiminase.

In a first study, normal vascular endothelial cells (HUVE), and neoplastic cells derived from two neuroblastomas (SH-EP, WAC2), a retinoblastoma (Y-79) and an osteosarcoma (SaOS) were used. All of them were inhibited in their growth when arginine deiminase was present, but the resulting cell cycle distributions were clearly different. The endothelial cells and cells from one of the neuroblastomas (SH-EP) showed negligible percentages in either the active or the inactive S-compartment. The other three cell types showed considerable quantities of unlabelled S-phase cells, with varying percentages in the other phases. These results were interpreted to mean that in addition to a simple depletion of arginine from the cellular microenvironment, arginine deiminase can also specifically modulate cell cycle progression. The details of the mechanisms involved, of course, remain to be elucidated, but it is important to note that as in the case of radiation, the cell cycle arrests observed did not appear to be correlated with apoptosis.

At the time of the study, the p53 status of these cell lines was not known. Since then it has been shown that the SH-EP line (Hopkins-Donaldson et al., 2002) is p53 wild-type, whereas SaOS is a p53 null cell line (DiBiase et al., 1999). This agrees with the observation that the former (like normal vascular endothelial cells) do not show unlabelled S-phase cells, whereas the latter do. The p53 status of WAC2 is not known. The line Y-79 is a p53 wild-type (Cobrinik et al., 2006), and thus does not seem to quite fit the picture, but as it stems from a

retinoblastoma, there are most probably other changes in the cell cycle regulation that have an influence on their progression through S-phase.

In a second study on arginine deiminase, similar investigations were carried out with two leukemia cell lines (Jurkat and Tanoue). Both showed a very clear accumulation of cells in the unlabelled S-compartment and a concomitant decrease of labelled cells. In this case, we also concomitantly observed apoptosis, which was assessed with two different methods: microscopic investigation of cell shrinkage and nuclear condensation on the one hand and end labelling of DNA breaks with terminal transferase (TUNEL) on the other. It is now known that Jurkat is a p53 mutant (Tíchy et al., 2008) and Tanoue is a p53 null cell line (Wissink et al., 2006), so the observation of unlabelled S-phase cells after treatment is not surprising.

4. Occurrence of quiescent S-phase cells under extreme physiological conditions

4.1 Low pH, hypoxia, and serum deprivation *in vitro*

F. Zölzer and C. Streffer, Radiation and/or hyperthermia sensitivity of human melanoma cells grown for several days in media with reduced pH. Strahlenther. Onkol. 175 (1999) 325-332

F. Zölzer, and C. Streffer, Sensitivity of human melanoma cells to hyperthermia and/or radiation: independence of intracellular pH. In: Radiation Research 1895-1995, Proceedings of the Tenth International Congress of Radiation Research, Ed. by U. Hagen, D. Harder, H. Jung and C. Streffer, Würzburg 1995, pp. 985-988

As briefly described in the introduction, indirect evidence of quiescent S-phase cells had been obtained with cell cultures kept under extreme physiological conditions such as low pH and hypoxia, at a time when the BrdU labelling technique was not yet commonly available. We therefore decided to extend our investigations from quiescent S-phase cells induced by irradiation and/or hyperthermia to those (possibly) caused by certain culture conditions. In contrast to the majority of earlier studies, incubation times were chosen to be not hours but days, so that they would more faithfully reflect the conditions under which cells exist in solid tumours, especially those at an advanced stage.

In a first series of experiments, we incubated human melanoma cells (MeWo) in media with pH values between 6.5 and 7.3. This was meant to simulate the interstitial pH found in many tumours *in vivo*, which can even be below 6.0, but is mostly between 6.5 and 7.0, whereas values between 7.0 and 7.5 are typical of normal tissue (Vaupel et al., 1989; Choi et al., 2013). Interestingly, the human melanoma cells used here did not show any change in the intracellular pH (measured with the help of 5(and 6)-carboxyfluorescein diacetate) even after up to 6 days of incubation in media with low pH. A value of 7.2 ± 0.2 was found in all cells irrespective of the extracellular pH. They seemed to possess a remarkably efficient proton transport system that enabled them to keep their internal H^+-concentration relatively low even in an acidic environment. Nevertheless, the cells did show changes in their sensitivity to irradiation and/or hyperthermia under the same conditions. At pH 6.5 instead of 7.3 in the medium, the surviving fraction after either treatment alone or both in combination was lower by a factor of 2 to 4. Other factors than the intracellular pH must therefore be involved.

As found in a second series of experiments, the same cells incubated at reduced pH for up to 6 days had quite a different proliferation pattern from those in normal medium. Although the overall form of their DNA histograms was almost the same, the BrdU labelling technique demonstrated that most of the cells with an S-phase DNA content were actually not progressing through the cell cycle any more (similar scattergrams as the ones obtained here are shown in Fig. 12).

The increased sensitivity of cells incubated in an acidic medium, however, could not be directly attributed to these cells, because they were obviously unable to re-enter the cell cycle: when the medium pH was first kept at 6.5 or 6.7 for some days and then changed back to normal, the percentage of unlabelled S-phase cells declined, but their absolute number remained constant with further incubation. They were thus most probably overgrown by cells in other phases of the cycle at the time of pH change.

F. Zölzer and C. Streffer, Increased radiosensitivity with chronic hypoxia in four human tumour cell lines. Int. J. Radiat. Oncol. Biol. Phys. 54 (2002) 910-920

Another extreme physiological condition prevailing in tumours *in vivo* is hypoxia. In the next series of experiments, we therefore incubated cells in an oxygen free atmosphere, and again in contrast to the majority of earlier studies did so for several days instead of only hours, in order

to simulate the situation of cells in poorly vascularized tumour regions. Because it had been reported that the p53 gene is induced under these conditions and in turn induces other genes (Graeber et al., 1994; Stommel and Wahl, 2005), we extended the study to four cell lines, namely the two melanoma and two squamous cell carcinoma lines mentioned above, each pair consisting of a p53 wildtype and a p53 mutant.

As in the case of low pH, we found that hypoxia did not dramatically change the overall form of the DNA histograms, but with some cell lines caused a reduction in the fraction of active S-phase cells, while the fraction of inactive S-cells increased. This was so with both p53 mutant cell lines, but did not show with the p53 wild types, either because they were unable to enter a quiescent state from the S-phase, or simply because they degenerated very quickly and their cell cycle distribution could not be analysed after longer incubation times.

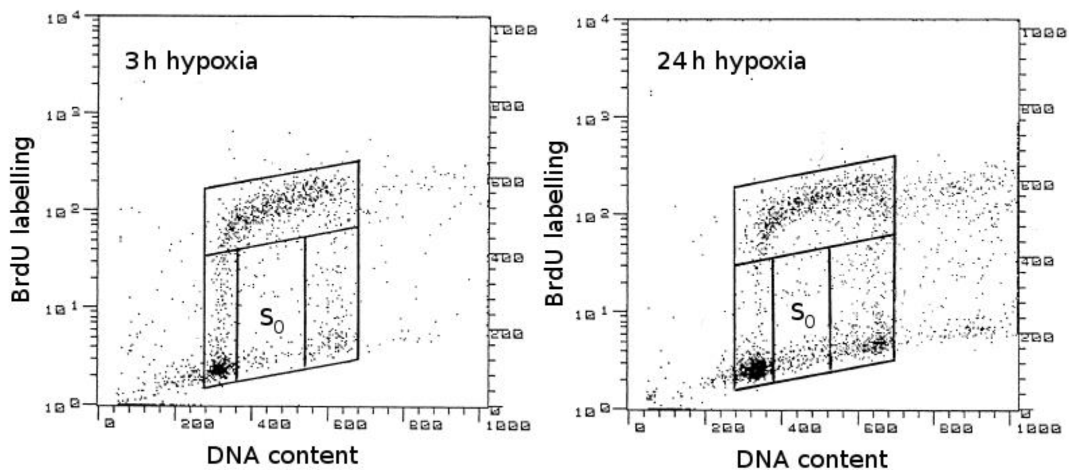

Fig. 4: Cell cycle distribution of human melanoma cells (MeWo) after 3 h or 24 h of incubation under hypoxia. Note how the number of quiescent S-phase cells (S_0) increases.

Similar to the experiments in which the pH of the medium was brought back to normal after some days, we incubated cells first under hypoxic conditions and then re-oxygenized them in order to see whether inactive S-phase cells would return to normal. This did not seem to be the case, because the fraction of inactive S-phase cells decreased no more than would be compatible with the assumption that they were unable to re-enter the cycle and were simply overgrown by proliferating cells.

Of great importance for clinical radiobiology is the fact that cells exposed to hypoxic conditions for a number of days became very sensitive to radiation. This is in contrast to the

well-known “radiobiological oxygen effect”, which consists in an increased resistance of (acutely) hypoxic cells in comparison with their oxygenated counterparts by a factor of 2 to 3. In the case of chronic hypoxia, this effect is obviously outweighed by the sensitization caused by other changes, probably metabolic ones which are also responsible for the observed changes in cell cycle progression. Quiescent S-phase cells thus seemed to present themselves as indicators of extreme physiological conditions influencing radiation sensitivity.

F. Zölzer, Radiation and/or hyperthermia sensitivity of human melanoma cells after several days of incubation in media lacking serum or certain serum components. Int. J. Radiat. Oncol. Biol. Phys. 46 (2000) 491-497

N. Oya, F. Zölzer, F. Werner, and C. Streffer, Effects of serum starvation on radiosensitivity, proliferation and apoptosis in four human tumor cell lines with different p53 status. Strahlenther. Onkol. 179 (2003) 99-106

Having investigated the effects of low pH and hypoxia on cells *in vitro*, it seemed natural to also look at other factors which might be scarce in certain types of tumours. Complete serum and single growth factors had been suggested in this context, and there were reports in the literature that they protected cells against the effects of hyperthermia, but no similar data were available for radiation (Langeland Marthinsen et al., 2002). Moreover, the phenomenon was not very well understood, especially with regard to the relative importance of the various serum components. We therefore developed a fully defined growth medium for the melanoma cells used in a number of earlier studies (MeWo), and then looked at the radiation and/or hyperthermia sensitivity of cells cultivated in media lacking one or the other component, such as insulin, transferrin, Na-selenite, and a lipid-protein complex, or medium not supplemented with either of these at all. In parallel, cell proliferation was characterized by the bromodeoxyuridine (BrdU) labelling index determined by two-parameter flow cytometry.

Radiation and/or hyperthermia sensitivity was hardly influenced by 3 days of incubation in medium containing no supplement at all, whereas after 6 days under the same conditions survival curves both of irradiated and hyperthermia-treated cells had a strongly reduced shoulder, indicating reduced ability to recover from sublethal damage. Experiments with media lacking only one or the other serum component showed that deprivation of insulin, transferrin, and Na-selenite led to no more than a modest increase in cell killing, whereas the

effects in medium lacking lipid-protein complex were about the same as in medium containing no supplement at all.

Sensitivity changes did not seem to be due to the lack of serum or certain of its components *per se*, but were very well correlated with changes in proliferation as characterized by BrdU labeling index. In particular, cells under conditions more strongly affecting sensitivities showed a reduced labelling index, and cells incubated in medium lacking lipid-protein complex or medium without any supplement at all even had significantly increased fractions of unlabelled S-phase cells (10 – 15 %, after 6 days instead of about 5 % with normal medium).

In a related study, we looked at the effects of serum starvation (medium with 0.5% fetal calf serum instead of 20%) on cells of different p53 status. Similarly to what was observed with medium lacking the lipid-protein complex or medium not containing any supplement at all, cells had a severely reduced growth rate, performing no more than 3 doublings within 3 to 5 days and then beginning to detach from the culture flask bottom. This degeneration was much faster with p53 wild types, as described above for hypoxic conditions. The changes in growth rate were mirrored in the BrdU labelling index, which generally decreased with time of culture in starvation medium, although only in the case of one squamous cell carcinoma (4197) it reached values below 5%. Surprisingly (in view of what has been reported so far), in all four cell lines the decrease in the labelling index was associated with an accumulation of quiescent S-phase cells. However, there were differences in the preferred state of quiescence, the ratio of the G_0/G_1-fraction to the unlabelled S-fraction after 6 days of starvation being larger for the p53 wild types (about 4.7) than for the p53 mutants (about 3.0).

As to the radiation sensitivity under these conditions, it was increased or remained unchanged with the two p53 mutants, but decreased with the p53 wild types, which is easily explained by the higher accumulation of cells in the G_0/G_1-phase. The details of the sensitivity changes, however, were difficult to interpret. For instance, the flow cytometric Annexin V assay showed that after 6 days of starvation the apoptotic fraction was 50% with Be11, but only 10% with 4197, and yet both cell lines showed similar changes in radiation sensitivity. Apoptosis did not occur in MeWo and 4151, while radiation sensitivity remained the same in one (MeWo) and increased in the other (4151). Additional factors, presumably p53 independent, seem to be at work here.

4.2 Tumour tissue *in vivo*

F. Zölzer, C. Streffer, and T. Pelzer, A comparison of different methods to determine cell proliferation by flow cytometry. Cell Prol. 27 (1994) 685-694

Although two-parameter flow cytometry with BrdU labelled cells seems to be the most reliable method available to assess the actual proliferation state of a cell culture or tumour, its application *in vivo* meets with problems. In particular, ethics commissions in some countries such as Germany have been reluctant to agree with the application of BrdU in patients. For the flow cytometric analysis of tumour biopsy material a single BrdU dose of about 200 mg m^{-2} has to be injected. This is far below the doses at which systemic effect on the bone marrow and skin of patients have been observed, namely 700 mg m^{-2} daily for about 2 weeks, but toxic and mutagenic risks cannot be excluded altogether. Therefore, alternatives to BrdU labelling of actively proliferating cells have to be considered. They became available through the development of monoclonal antibodies against proliferation associated molecules such as PCNA (proliferating cell nuclear antigen) or the Ki-67 antigen. PCNA functions as a co-factor of polymerase δ and under specific conditions may be used to identify S-phase cells (Sasaki et al., 1993; Scovassi and Prosperi, 2006). The function of the Ki-67 antigen remains as yet unclear, but it is widely accepted as a marker of cycling (G_1, S, G_2) as opposed to resting (G_0 and others) cells (Danova et al., 1988; Scholzen and Gerdes, 2000). By the middle of the 90s, both of them had been used in two-parameter flow cytometry, but to what extent the results obtained were comparable with those from investigations involving BrdU labelling remained to be clarified. We therefore undertook a number of *in vitro* experiments which compared PCNA and Ki-67 expression on the one hand with BrdU labelling on the other.

Melanoma cells (MeWo) at different culture ages were compared. The BrdU pulse labelling index agreed very well with the fraction of PCNA positive cells, provided the cells had been treated with pepsin, i.e. only cell nuclei were measured. With whole cells, there was an almost uniform expression of PCNA throughout the cell cycle. Only in plateau cultures a tendency

towards reduced expression in the $G_{0/1}$ compartment became visible, but it was impossible to reliably separate two subpopulations. The conclusion was that the PCNA protein had a long half-life, but that the fraction bound to nuclear structures varied during the cell cycle and therefore could be used to identify S-phase cells.

In order to measure the growth fraction of a cell culture, the BrdU pulse labelling method is obviously inappropriate. Continuous labelling experiments for periods of up to 36 h were therefore undertaken, and it was found that the labelling index reached a saturation level after about 24 h. The final BrdU labelling index was then compared with the fraction of Ki-67 positive cells and not surprisingly the two parameters agreed quite well. In contrast to the PCNA measurements, the cycling and quiescent subpopulations could be clearly separated in two-parameter flow cytometry. Thus, for whole cells, and where the fraction of cycling and quiescent cells is to be assessed, Ki-67 seemed to be the appropriate marker.

F. Zölzer, A. Speer, T. Pelzer, and C. Streffer, Evidence for quiescent S- and G_2-phase cells in human colorectal carcinomas: a flow cytometric study with the Ki-67 antibody. Cell Prolif. 28 (1995) 313-327

The study of proliferation patterns in colorectal tumours was then continued with the analysis of material from biopsies of human tumours. We studied proliferation patterns with the help of two-parameter flow cytometry, using the Ki-67 antibody to distinguish between cycling and non-cycling cells as had been done in the *in vitro* experiments just described. The cut-off between Ki-67 positive and negative subpopulations was unequivocal in most cases, but in some could only be set using an appropriate blank antibody as control. Nevertheless, it was clear that in a number of cases non-cycling cells were present not only in the $G_{0/1}$ compartment, but also at an S- or G_2-phase DNA content.

Not unexpectedly, when the DNA histograms of the cycling and the non-cycling subpopulations were subjected to cell cycle analysis separately, the fraction of S- and G_2-phase cells turned out to be smaller in the Ki-67 negative than in the Ki-67 positive subpopulation, even if this was only by about 1/3 on average. Thus, we considered the reports of other authors on quiescent cells in all cell cycle phases as corroborated.

Most importantly, when the (S+G_2)-phase fraction of the Ki-67-negative subpopulation was compared in tumours of different sizes (T2, T3 and T4), a significant trend at $p<0.05$ was seen, larger tumours showing higher fractions of non-cycling cells in the S- and G_2-phase.

This was even more interesting as none of the other parameters investigated showed any dependence on the general tumour stage (Duke's A, B, C or D): neither the Ki-67 index or the

fraction of S- and G_2-phase cells of the total population, nor the $(S+G_2)$-phase fraction of the Ki-67-negative subpopulation. The fact that it was the tumour size that mattered seemed to support our hypothesis that the physiological conditions prevailing in larger tumours, especially hypoxia and low pH, cause cells to leave the cell cycle in phases other than $G_{0/1}$.

F. Zölzer, G. Stüben, K. Knühmann, C. Streffer and H. Sack, Quiescent S-phase cells as indicators of extreme physiological conditions in human tumor xenografts. Int. J. Radiat. Oncol. Biol. Phys. 45 (1999) 1019-1024

In order to test the said hypothesis more directly, we studied the possible correlation between the frequency of quiescent S-phase cells and the oxygenation in human tumour xenografts on nude mice. Oxygenation was measured polarographically with a needle electrode in 24 to 30 individual tumours each of five different entities. In parallel, mice were injected with BrdU, tumours were excised 30 min later and two-parameter flow cytometry as described above was carried out for cell cycle analysis. The frequency of measuring points with oxygen partial pressure <5 mm Hg was between 70 and 100 %, typical values of the frequency of unlabelled S-phase cells were between 5 and 20 %, and both parameters increased with tumour volume. There was a highly significant correlation ($p<0.002$) between the two, showing that in more hypoxic tumours a greater fraction of S-phase cells had gone into a quiescent state.

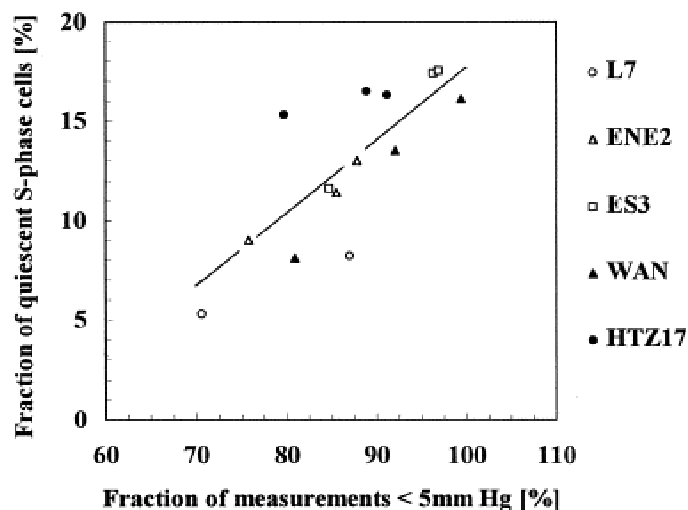

Fig. 5: Relationship between tumour oxygenation and the occurrence of quiescent S-phase cells in five tumour entities. For each entity, tumours of three different sizes were compared.

It could be argued, of course, that the BrdU used as label did not reach cells in poorly vascularized areas and that as a consequence an indirect relationship between oxygenation and the frequency of unlabelled cells appeared. This explanation, however, seemed unlikely for a number of reasons, most importantly because microscopical inspection of tumour sections showed that the BrdU label did indeed reach hypoxic areas (which in these cases were identified with the help of pimonidazole injected at the same time as BrdU).

It is thus most probable that the inactive S-phase cells observed in human tumour xenografts are not artefacts, and the correlation between their frequency and the degree of oxygenation is due to a direct influence of the tumour micromilieu on cell cycle regulation. Judging from our *in vitro* studies, it seems likely that this influence requires a few days to be established, so that only chronic hypoxia, occurring in large tumours which have outgrown their vasculature (but not acute hypoxia, which is due to a temporary occlusion of blood vessels) would be expected to yield this kind of results.

5. Prospects for future fundamental and applied research

The studies presented above relate to disturbances of tumour cell proliferation caused by external factors, mainly by radiation, but also by hyperthermia (alone or in combination with radiation), by chemical growth inhibitors or by physiological conditions which are typically found in tumours, in particular low pH, hypoxia, and serum deprivation. The results obtained are therefore of primary interest in the context of clinical radiobiology, and more particular with regard to the individualization of tumour therapy. It has become more and more obvious in the course of the last few decades that human tumours, even if they are of the same stage and histopathological classification, react very differently to treatment. This is based on a strong interindividual as well as intraindividual heterogeneity. A number of factors have been studied in this respect, such as intrinsic cellular radiosensitivity (Fertil and Malaise, 1985; Darzynkiewicz et al., 2009), cell proliferation and cell loss (Sager, 1990; Williams et al., 2008a), and the tumour micromilieu (Vaupel et al., 1992; Yaromina et al., 2009). At least these three have been clearly shown to exert an influence on treatment response, and unless we characterize and learn to understand their respective mechanisms of action, we cannot expect to become able to first predict and then improve therapy success.

It would seem that flow cytometry has an enormous potential for the characterization of proliferation patterns in human tumours, which has not yet been completely realized. The exact distribution of cells to the different cell cycle compartments, the dynamics of their movement through the phases of the cycle, the influence of physiological conditions as well as physical and chemical factors, and last but not least the genetic set-up of the cells – all this may have great importance on the reaction of tumours to therapy. Therefore, two-parameter flow cytometry will surely be of increasing importance in the future.

Cell cycle dynamics after radiation exposure or exposure to hyperthermia were here studied with a view on just one genetic determinant, namely p53. Even though this gene seems to be one of the major players in many cases, dozens of other genes have been identified which also have an influence on cell cycle checkpoints (Rai et al., 2007). Their respective roles and their interactions would need to be studied in a similar way as p53 was studied here.

The spectrum of physiological conditions which we have considered could also be broadened. Tumour areas with reduced vascularization are characterized not only by hypoxia, a reduced pH and an insufficient supply with growth factors and other critical proteins and lipids, but also by a lack of glucose and other sources of energy such as glutamine (DeBerardinis and Cheng, 2010). Preliminary experiments suggest that these, too, exert a strong influence on proliferation patterns, but that their relative importance varies from cell type to cell type (Streffer, 1994; Feron, 2009). It is clear, moreover, that the different factors contributing to the tumour micromilieu have different diffusion behaviour, i.e. they reach to different depths from the supplying blood vessels (Erickson et al., 2003). Therefore, a tumour may contain areas which are completely lacking in oxygen, growth factors and energy sources, but there may be others which are hypoxic while still provided with glucose, leading to anaerobic metabolism of tumour cells, or still others which have neither oxygen nor glucose, but a different energy source which is less efficiently used, but nevertheless allows for continued tumour cell growth.

Thinking more closely about the prospects for the clinic which the methods presented above may offer, one has to say that the study of cell cycle dynamics *in vivo* presents certain problems. Begg et al., 1985, suggested their method as one that would allow the determination of S-phase duration and potential tumour doubling time from just one biopsy

(taken a certain number of hours after injection of BrdU). However, the quantitative analysis of cell cycle progression, especially the determination of cell cycle phase durations and their changes due to radiation and other factors as suggested here, needs data for a number of time points. As it is not feasible to take multiple biopsies of patients, these methods cannot really be applied in the clinic. One could think of *ex vivo* short term tissue cultures, but our further studies pointing to the critical importance of the physiological conditions surrounding the cells make such an approach questionable in clinical tumours – as valuable as they might be for the elucidation of cell cycle dynamic cell culture or animal experiments.

However, the phenomenon of quiescent S-phase cells, which has been largely ignored so far, should definitely receive greater attention in the clinic. It can be taken into account without the need for multiple biopsies, but the information thus gained will be highly valuable. A few years ago, one of the doctoral students involved in our research program conducted a study into the predictive potential of the S-phase fraction for the radiation therapy of brain tumours. He showed that those patients with higher proliferative activity did not profit at all from post-operative irradiation (Farahani, 2003). If this could be confirmed in general, as many as half of all brain tumour patients could be spared the trauma of an additional radiation therapy. However, as only classical DNA histograms were analysed in that study, it is necessary to revisit the question with a more complete analysis of proliferative patterns including differentiation between cycling and non-cycling S-phase cells. And then of course similar studies will have to be carried out for other tumour entities, where again the pre-treatment proliferative state may help to identify patients who would profit from additional irradiation and those who would not.

This latter example shows that the methods presented here have both more fundamental aspects, such as understanding the influence of genetic factors and physiological conditions, as well as more applied aspects, such as developing predictive assays, which could reach clinical relevance within a relatively short time. Close collaboration is needed in this field between specialists of biophysics, molecular biology, radiation biology, radiation therapy, and general oncology.

Resumé

The studies presented here refer to disturbances of tumour cell proliferation caused by external factors, mainly by radiation, but also by hyperthermia (alone or in combination with radiation), by chemical growth inhibitors, or by physiological conditions which are often found in tumours, particularly low pH, hypoxia, and serum deprivation. Such disturbances of proliferation play an important role for the individual tumour's response to treatment, and unless we characterize and learn to understand the respective mechanisms of action, we cannot expect to become able to first predict and then improve therapy success. The method of choice for these investigations is two-parameter flow cytometry, which combines the measurement of DNA content with the detection of a cellular function, DNA synthesis.

In a first series of studies, the movement of cells through the cell cycle after suffering DNA damage was analysed *in vitro*. We confirmed and extended earlier findings that the transition from G_1 to S is closely regulated by the p53 protein. It was also shown that p53 has a decisive influence on the progress through the S-phase, which was hitherto unknown. When we studied the movement of cells through G_2 and M into the next G_1 phase it became obvious that contrary to widespread assumptions, p53 is irrelevant in this case.

In a second series of investigations, we observed the induction of quiescent cells in the S-phase by physical and chemical factors. Irradiation and/or hyperthermia, as well as a chemical growth inhibitor, arginine deiminase, caused an increase in the fraction of cells which had a DNA content typical of the S-phase but were nevertheless not actively synthesizing DNA. This phenomenon had been observed before, but had not yet been characterized in detail. We were able to show that it does not occur in all cells, but is dependent on the functionality of the p53 protein: quiescent S-phase cells are only induced in p53 mutants, i.e. in cells which are unable to activate the G_1 checkpoint and have therefore less time available for DNA repair before the start of replication.

The third block of studies concerned quiescent cells in the S-phase under extreme physiological conditions *in vitro*, particularly low pH, hypoxia, and serum deprivation. These were chosen because they reflect the situation in a solid tumour, especially at an advanced stage when the tumour has outgrown its vasculature. All three conditions, if they lasted for several days, caused the transition of cells into a quiescent S-phase state. Earlier indications

that this was happening had not been followed up by other authors. Our studies suggested that quiescent S-phase cells can be considered as indicators of those physiological conditions which cause cells to be sensitive to treatment modalities such as radiation and/or hyperthermia. That such cells also exist *in vivo* is supported by findings in human colorectal tumours, and especially in xenografts of human tumours on mice, where the occurrence of quiescent S-phase cells was shown to be significantly correlated with the extent of hypoxia.

In summary, it would seem that the methods presented here have a great potential for the characterization of proliferation patterns in human tumours. The exact distribution of cells to the different cell cycle compartments, the dynamics of their movement through the phases of the cycle, the influence of physiological conditions as well as physical and chemical factors, and last but not least the genetic set-up of the cells – all this has great importance on the response of tumours to therapy. Investigations into the cell cycle disturbances induced by radiation and other cellular stresses will surely be of increasing importance in the future.

Resumé (český)

Předkládané studie se zabývají poruchami proliferace nádorových buněk vyvolanými vnějšími faktory, a to zejména zářením, ale též hypertermií (buď samostatně, nebo v kombinaci se zářením), chemickými inhibitory růstu a nebo fyziologickými změnami, které se u nádorů často vyskytují, jako je nízké pH, hypoxie a nedostatek séra. Tyto proliferační poruchy mají významný vliv na reakci nádoru a na průběh léčby, a pokud neanalyzujeme příslušné mechanismy účinků a neporozumíme jim, nelze predikovat úspěšnost terapie a následně tuto úspěšnost dále zvyšovat. Vhodnou metodou u těchto druhů výzkumů je dvouparametrová průtoková cytometrie, která propojuje snímání obsahu DNA s detekcí buněčné funkce, syntézy DNA.

V první sérii studií byl analyzován průchod buněk *in vitro* buněčným cyklem po poškození DNA. Potvrdili a doplnili jsme dřívější závěry, že přechod z G_1 do S fáze buněčného cyklu je regulován proteinem p53. Bylo také prokázáno, že p53 má rozhodující vliv na přechod přes S-fázi, což nebylo dosud známo. Při sledování přechodu buněk z G_2 a M fáze do další fáze G_1 bylo zřejmé, že na rozdíl od rozšířených předpokladů je zde funkce proteinu p53 irelevantní.

V dalších studiích jsme pozorovali indukci buněk v klidové S fázi fyzikálními a chemickými faktory. Ukázalo se, že záření a/nebo hypertermie a též inhibitor růstu arginin deiminase způsobuje zvýšení frakce buněk s obsahem DNA typický pro S fázi, které se nicméně nepodílí na syntéze DNA. Tento jev byl pozorován dříve, ale ještě nebyl detailně charakterizován. Prokázali jsme, že se nevyskytuje u všech buněk, ale je závislý na funkci proteinu p53: klidové buňky v S-fázi se vytváří pouze u p53 mutantů, tedy v buňkách, které nejsou schopny aktivovat checkpoint v G_1 a mají proto méně času k opravě DNA před začátkem replikace.

Třetí blok studií se týkal buněk mimo cyklus za extrémních fyziologických podmínek, jako je nízké pH, hypoxie a nedostatek séra. Tyto parametry byly zvoleny z důvodu jejich podobnosti v mnoha solidních nádorech, zejména v jejich pokročilém stádiu, kdy nádor přerostl svou vaskulaturu. Všechny tři uvedené podmínky při několikadenním trvání způsobovaly přechod buněk do klidové S fáze. Dřívější náznaky, že k tomu dochází, nebyly sledovány jinými autory. Naše studie naznačují, že klidové buňky v S-fázi mohou být považovány za ukazatele takových fyziologických podmínek, které způsobují, že buňky jsou citlivé na způsoby léčby,

jako je záření a/nebo hypertermie. Existenci těchto buněk *in vivo* podporují výsledky na lidských kolorektálních nádorech a zejména v xenograftech lidských nádorů na myších. Zde výskyt klidových buněk v S-fázi významně koreluje s rozsahem hypoxie.

Na závěr jsme dospěli k tomu, že zde uvedené metody mají velký potenciál pro charakterizaci proliferace buněk v lidských nádorech. Přesné rozdělení buněk do různých fází buněčného cyklu, dynamika jejich pohybu v průběhu jednotlivých fází cyklu, vliv fyziologických podmínek, jakož i fyzikálních a chemických faktorů, a v neposlední řadě i genetické nastavení buněk - to vše má velký význam na reakci nádorů na léčbu. Do budoucna bude mít stále větší význam analýza poruch buněčného cyklu vyvolaná zářením a dalšími buněčnými stresy.

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