4. DISCUSSION

Studies with cell lines serve as an initial screen for searching for different modalities to treat cancer and reveal potential mechanisms of interaction between drugs, but also between the different therapeutic options. Understanding the mechanisms, which lead to the multidrug resistance phenomenon, may improve the clinical outcome in cancer patients. We evaluated systematically the expression of the three main factors of multidrug resistance - mdr1/P-glycoprotein, mrp1/MRP1 and lrp/LRP on gene as well on protein level and tried to extrapolate these data on chemosensitivity assays with schedules of cytotoxic drugs, which are commonly used in clinical practice. We explored the relationship between radiation and chemotherapy in terms of development of chemoresistance after irradiation.

4.1. Relationship between mdr1/P-glycoprotein expression and radiation therapy

P-glycoprotein is the prototype of the ABC-transporters encoding drug resistance and causes chemoresistance of diverse cancers and cancer cell lines (Gottesman, 2002). Recent studies have reported that irradiation of tumor cell lines is associated with overexpression of P-glycoprotein (Bu et al., 2005; Hill et al., 2000+2001). Hill et al., 2001, who treated Chinese hamster ovary tumor cells (CHO) with 9 Gy x 10 or a single dose of 30 Gy and human tumor ovarian cells with 10 Gy x 5, found elevated levels of P-glycoprotein. The observed upregulation of P-glycoprotein expression by different chemotherapy regimens and fractionated or single dose radiotherapy suggests that the activation of the ABC-transporter might be based more generally on cellular stress responses.

The multidrug resistant cell lines derived after in vivo or in vitro incubation with cytotoxic drugs are characterized with an increased mdr1 gene copy number, mRNA content and protein product (Chion et al., 2003). However, after completion of irradiation treatment no increased expression of mdr1 mRNA was reported. Moreover, Henness et

al., 2004, demonstrated that P-glycoprotein overexpression could occur despite mdr1 mRNA downregulation. They proved that this phenomenon is due to an increased protein stability and half-life with corresponding decreases in turnover rates after irradiation. The exact mechanism of this process remains unknown, though recently, new data have provided a possible connection between P-glycoprotein upregulation and the activity of the proteasome cleavable complex (Fujita et al., 2005). In the present study, we found a limited expression of P-glycoprotein in chemo- and radio-naive cell lines, supporting previous studies, which suggested that P-glycoprotein expression is of minor relevance to the intrinsic therapy resistance of cancer (Berger et al., 2005). We found overexpression of mdr1 mRNA near the level of statistical significance in a group of five breast cancer cell lines three days after the last irradiation with 27 Gy. However, in the group of colon cancer cell lines no significant mdr1 mRNA overexpression was detected on day 3 and on day 18 after the last irradiation. Our data imply a new possible mechanism of P-glycoprotein overexpression, namely that it could be due not only to an increased protein stability and half-life with corresponding decreases in turnover rates, but in certain cell lines also to an mdr1 gene upregulation (3.2). However, our results do not contradict to previous investigations, because for the first time the highly accurate method of real-time PCR was used to assess the gene expression following radiotherapy. In agreement with previous results, we demonstrated a highly significant upregulation of P-glycoprotein among eleven breast and colon cancer cell lines. Moreover, we found a connection between P-glycoprotein and mdr1 mRNA level in five breast cancer cell lines. Interestingly, the breast cancer cell lines, in which a significant mdr1 gene upregulation was found, had an albeit small P-glycoprotein upregulation. We assume that in the colon cancer cell lines, investigated by us, increased P-glycoprotein half-life after irradiation rather than mdr1 gene upregulation is more probable. The breast cancer cell lines, however, seem to upregulate the P-glycoprotein by both mechanisms.

4.2. Relationship between mrp1/MRP1 expression and radiation therapy

MRP1, another ABC transporter, is now recognized as a pivotal factor in the development of multidrug resistance in tumors before as well as after chemotherapy. MRP1 was first cloned from a drug selected SCLC cell line (Cole et al., 1995) and acquired and intrinsic drug resistance was attributed to it (Young et al., 2001). Expression of MRP1 in vitro correlated with enhanced resistance to a broad spectrum of MRP1 substrate drugs and especially to doxorubicin (Davey et al., 1996; Wyler et al., 1997). However, possible MRP1 participation in the multidrug resistance following gamma-irradiation has been recently implicated and there is insufficient information about the mechanisms of MRP1 upregulation (Henness et al., 2001; Hill et al., 2000). A six-fold increase in mrp1 mRNA levels has been reported in a human T-cell leukemia cell line after 75 Gy (Harvie et al., 1997), which was explained by gene amplification or mutation; yet, mechanism of MRP1 overexpression similar to that in P-glycoprotein following irradiation could not be ruled out (Hill et al., 2000). We report a significant mrp1 mRNA overexpression in a group of five breast cancer cell lines, 3 days after completion of the irradiation treatment; however, such overexpression was not observed in six colon cancer cell lines. Nonetheless, a significant MRP1 overexpression occurred in the breast and colon cancer cell lines following fractionated low-dose radiation treatment, but there was no correlation between mrp1 mRNA and MRP1 level. Our results suggest that there could exist two possible different pathways of MRP1 overexpression: gene amplification, which was demonstrated in the breast cancer cell lines and increase of protein stability and half-life, observed in the colon cancer cell lines. Furthermore, we propose that the mRNA and protein co-expression, typical for the multidrug resistance phenomenon following chemotherapy, could be a feature of the resistance after radiation treatment. To our knowledge, the co-expression of mdr1 and mrp1 mRNA observed in this study, is the first evidence of fractionated irradiation increasing both the expression level of the genes of these two ABC transporters. It was reported that MRP1 overexpression after irradiation was observed in cancer cells, which did not overexpress P-glycoprotein, suggesting that either P-glycoprotein or MRP1 was involved in the multidrug resistance. However, in some cell lines we observed a simultaneous upregulation of both P-

glycoprotein and MRP1. In two lines, HCT116 and SW403, the MRP1 upregulation was steady during the entire investigation period and reflected their drug sensitivity profile.

4.3. Relationship between lrp/LRP expression and radiation therapy

The lung resistance protein (LRP) is associated with the occurrence of chemoresistance and is recognized as a negative prognostic factor for the therapeutic response (Mossink et al., 2003). Vaults, judged by the LRP expression, closely reflect the chemoresistance profile of many tumor cell lines and cancers and elevated levels of LRP were observed in cell lines resistant to various cytotoxic drugs (Schroeijers et al., 2000; Sugawa et al., 1997). To our knowledge, no study has reported a possible implication of lrp/LRP in the drug resistance appearing after completion of radiation therapy. However, we observed a significant overexpression of LRP three days after the last irradiation of 27 Gy, which was not accompanied by a significant lrp gene upregulation. The LRP overexpression was significant in the colon cancer cell lines as well as in the breast cancer cell lines, but at a lower level. We suppose that LRP increased stability and half-life with corresponding decreases in turnover rates, rather than gene amplification, could be the cause of the protein overexpression after irradiation. LRP seems to be a major factor in the response to environmental stimuli, thus contributing to the occurrence of a multidrug resistance phenotype after radiation treatment, independently of MRP1 and P-glycoprotein. However, it was transient and hence could not play an important role in the long-term multidrug resistance phenotype, which persisted autonomously when LRP overexpression was not present any more.

4.4. Multidrug resistance following radiation therapy

Cellular drug resistance is a major reason for failure of treatment. The classic multidrug resistance phenotype is a well-characterized phenomenon of expression of ABC transport proteins, which confer resistance to anthracyclines, but not to cisplatin (Salerno et al.,

2004). On the other hand, cisplatin resistance is often reported after irradiation (Caney et al., 1999+2004; Eichholz et al., 1993). LRP, the major vault protein, is reported to confer resistance to various non-related compounds including anthracyclines and cisplatin (Kartalou et al., 2001). Fractionated radiation has been reported to cause drug resistance in ovarian cells, CHO, ascites tumor cell, NSCLC (non-small cell lung cancer) and human T-leukemia cell lines. However, the few cell models used in previous studies applied a fractionation procedure completely different from that used in the clinic. Thus, although the total dose of irradiation administered in vitro was within that specified in clinical protocols, the results obtained could be strongly influenced. In addition, no study has attempted to estimate if multidrug resistance occurs at low doses of gamma-irradiation. This fact would have significant implications in the modern therapeutic schemes, which are based on simultaneous application of chemotherapy and radiotherapy. To our knowledge, no investigations have been carried out concerning the relationship between radiation therapy and multidrug resistance in colorectal cancer cell lines.

In our study, we report of resistance to doxorubicin, cisplatin and bendamustine three days after completion of low-fractionated irradiation with 27 Gy. Combining the protein data and functional results of breast and colorectal cancer lines revealed a significant correlation between expression of P-glycoprotein and MRP1, and on the other hand resistance to doxorubicin and bendamustine. Expectedly, such a correlation was not found for resistance to cisplatin. This imposes the ‘classic’ P-glycoprotein/MRP1 resistance as the main factor of susceptibility of the cancer cell to bendamustine and doxorubicin. The observed correlation further supports the relevance of radiation-induced P-glycoprotein expression for drug resistance, as doxorubicin, in contrast to cisplatin, is a substrate for both membrane transporters. Blocking studies with verapamil did confirm this mechanism in one colon cancer cell line. We detected a high correlation (3.10) between LRP expression and resistance to doxorubicin on day 18, which was independent of the ‘classic’ one. It seems that P-glycoprotein and MRP1 conferred resistance to doxorubicin and bendamustine after irradiation and hint to a possible similarity of the resistance mechanisms to doxorubicin and bendamustine. Some authors reported that MRP1 can act as a GS-X pump and as such could participate in the

detoxification of heavy metals, i.e., platinum (Kruh et al., 2003). However, we did not detect any correlation between resistance to cisplatin and the overexpression of P-glycoprotein, MRP1 and LRP. We know that cell growth could influence the sensitivity profile of cancer cells to drugs. To reduce that factor, we allowed five colon cancer cell lines to regain logarithmic growth and assessed their sensitivity to cytotoxic drugs. We observed a low, but significant resistance to doxorubicin and cisplatin, which was accompanied by P-glycoprotein overexpression. Two cell lines retained a high level of MRP1 and this was complemented with significant resistance to doxorubicin and bendamustine, but not cisplatin. The last fact proves the existence of multidrug resistance phenomenon after irradiation – appearance of resistance to structurally and functionally unrelated cytotoxic drugs.

However, cell growth remained a possible important factor in the multidrug resistance phenotype after radiation therapy – on day 18, Colo320 colon cancer cell line had a considerably slower growth rate than the non-irradiated parallel control cultures and was still resistant to all three cytotoxic drugs, although it did not overexpress any of the three studied proteins.

4.5. Modulation of the multidrug resistance phenotype following irradiation using the calcium channels blocker verapamil

There are studies attributing to verapamil and other calcium channel blockers multidrug resistance modulating properties (Salerno et al., 2004). Modulation as part of the initial therapy may prevent subsequent emergence of ABC protein-mediated resistance. However, there is no study to determine if such modulators could overcome the irradiation-associated multidrug resistance in breast and colon cancer tumor cells. To determine the relevance of the different multidrug resistance mechanisms following irradiation verapamil, a potent P-glycoprotein inhibitor, was used in two non-toxic doses; moreover, the highest dose used is known to block MRP1 activity, too (Takara et al., 2002). A poor chemosensitizing effect was observed on day 3, which may be due to the growth arrest following irradiation. However, we observed an enhancement of the

doxorubicin sensitivity in the Caco2 colon cancer cell line, a line notably overexpressing P-glycoprotein on day 18. However, the sensitivity to bendamustine and cisplatin remained unchanged. The blocking experiments with verapamil validated the significance of P-glycoprotein for the multidrug resistance phenomenon after radiation therapy. It remains unclear why some cells could evade apoptosis induction after co-incubation with verapamil, even though they overexpress P-glycoprotein. A possible explanation could be the concurrent LRP overexpression or cell growth arrest.

4.6. Conclusion

In the present study we assessed the appearance of multidrug resistance in cell lines of the two most common tumors treated with a combined radio-chemotherapeutic method: breast and colorectal cancer. We confirm and extend the findings of previous small-scale studies that irradiation could cause drug resistance to chemically unrelated drugs. Drug resistance appeared during radiation treatment, which mimicked the clinical situation at relatively low doses. This resistance was accompanied by overexpression of two ABC proteins, namely P-glycoprotein and MRP1, and persisted during the treatment-free period. It was also accompanied by mrp1 mRNA and mdr1 mRNA upregulations, introducing the mdr1 mRNA increase as a possible new mechanism in the P-glycoprotein overexpression. Eventually, LRP overexpression without concomitant lrp gene upregulation occurred as part of the cell response to environmental stimuli that might contribute to the acute post-irradiation multidrug resistance. In the investigated irradiated cancer cell lines a statistically significant increase of the cell resistance to physiological and higher concentrations of cisplatin, doxorubicin and the novel cytotoxic drug – bendamustine was observed compared to that in the non-irradiated controls (3.5 and 3.9). The correlation between the expression of P-glycoprotein, MRP1 and resistance to doxorubicin confirmed previous findings (Hill et al., 2001); however, our results implicate an analogous mechanism of resistance to structurally unrelated drug – bendamustine. The post-irradiation resistance to cisplatin was possibly determined not by the expression of MRP1 and P-glycoprotein as it correlated poorly with the expression of

these proteins. A novel finding of our study was the induction of LRP overexpression, which partially matched with lrp mRNA upregulation. LRP overexpression may in part explain radiation-induced chemoresistance to cisplatin, as LRP expression has been linked to resistance to a number of agents, including cisplatin. However, no significant correlation was observed between LRP overexpression and resistance to cisplatin. The correlation between the LRP expression and the resistance to doxorubicin was fairly independent of the P-glycoprotein/MRP1 and was detected 18 days after the last irradiation in colon cancer cell lines. The multidrug resistance phenomenon was observed at low doses of gamma-irradiation (27 Gy) and could be a possible explanation of the lower rate of response to chemotherapy associated with previous radiotherapy, but may also render cancer less sensitive to the concurrent use of chemotherapeutic drugs and irradiation. In that sense we suppose that the concurrent use of chemotherapeutic drugs in the second half of the radiotherapeutic protocol should be reconsidered and in vivo investigations are needed in that area. The drug resistance was poorly modulated by P-glycoprotein inhibitors, which may indicate a secondary role of the P-glycoprotein in the multidrug resistance phenotype after irradiation in the investigated cell lines. However, additional studies are considered necessary to shed light on the actual role of P-glycoprotein, MRP1 and LRP overexpression after radiation therapy. Other mechanisms of the reduced tumor sensitivity to drugs should be considered, as none of the reported so far could explain the multidrug resistance phenomenon, which was still present despite the lack of P-glycoprotein, MRP1 and LRP overexpression or cell growth arrest. The basic finding of our investigation is that breast and colorectal cancer cells surviving fractionated irradiation frequently display expression of MDR-associated molecules and functional chemoresistance. It is unclear, whether the multidrug resistance phenotype was actually induced by irradiation, or whether the surviving cancer cells happened to belong to a small constitutively resistant subset of the cell lines. The last is, however, unlikely because of the homogenously low expression of multidrug resistance-related molecules in non-irradiated cell lines, and because of the at least partial reversal of the phenomena within 18 days after completion of irradiation.