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IMPAIRMENT OF LIVER REGENERATION DURING INHIBITION OF MITOCHONDRIAL PROTEIN SYNTHESIS BY OXYTETRACYCLINE

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Under standard conditions, liver regeneration is not impaired if mitochondrial protein synthesis is completely blocked. By treating rats with oxytetracycline for various periods of time directly prior to partial hepatectomy, livers were led to a condition of relative deficiency in cytochrome *c* oxidase and ATP synthetase. To this end, oxytetracycline was administered by means of continuous intravenous infusion up to concentrations of 20 μg/ml serum, giving a gradual decrease in cytochrome *c* oxidase activity. This activity was used as a marker for functionally capable mitochondria and as a tool to monitor the efficiency of inhibition of mitochondrial protein synthesis. It is shown that liver regeneration is strongly impaired after a period of pretreatment of 22 days or more and continuation of oxytetracycline treatment during regeneration. The mitochondrial respiratory capacity is reduced to 14% of the control value under these conditions. To obtain inhibitory levels within the regenerating liver, it was necessary to raise the serum levels slightly above 20 μg/ml. This measure is most likely required because of the poor vascularization of the regenerating liver. The serum levels were kept, however, far below those known to inhibit cytoplasmic protein synthesis. The results show that in normal liver the respiratory capacity must be reduced drastically before energy-requiring processes become affected. In Zajdela hepatoma cells, similar effects are found after reduction of the cytochrome *c* oxidase activity to 38%. This difference in sensitivity is probably based on the different mitochondrial content of liver cells and the liver-derived Zajdela cells.

Introduction

It is well known that the mitochondrial genome codes for a number of hydrophobic polypeptides, which are all part of enzyme complexes involved in oxidative phosphorylation (cytochrome aa_3, cytochrome bc_1 and ATP synthetase [1]). The genes involved are transcribed and translated within the mitochondrion itself. If mitochondrial protein synthesis is continuously blocked, the amount of the electron carriers cytochromes aa_3 and bc_1 will gradually decrease. Because the ATP synthetase is partly mitochondrially made, impairment of mitochondrial protein synthesis will also affect the ATP-synthesizing capacity as such. Prolonged inhibition of mitochondrial protein synthesis can thus result in reduction of ATP-generating capacity by diminution of the number of functional respiratory chains as well as of the amount of functional ATP synthetase. For these reasons a deficit of ATP leading to impairment of energy-requiring processes is finally to be expected.

Mitochondrial protein synthesis in vivo can be blocked by a number of antibiotics, e.g., oxytetracycline. A substantial reduction of the amount of mitochondrially made polypeptides will, however, only be found if the experimental period lasts long enough to allow passage of at least one cell cycle or one turnover of the cytoplasm of the cells in the tissue studied. For this reason, several authors

investigated the effect of impairment of mitochondrial protein synthesis on rapidly proliferating tissues, such as regenerating liver [2] or intestinal epithelium [3,4]. From the results obtained, it became clear that reduction of the amount of cytochrome aa_3 (either measured spectrally or as the specific cytochrome *c* oxidase activity) to at least 50% of the control value has no serious functional drawbacks. Liver regeneration, for instance, proceeds normally in spite of continuous inhibition of mitochondrial protein synthesis. Because a process like liver regeneration demands undoubtedly a lot of energy, this implies that somehow a large reserve capacity for ATP regeneration exists.

On the other hand, we have shown that prolonged inhibition of mitochondrial protein synthesis leads to cell proliferation arrest in two tumor cell systems in vivo [5,6]. It has to be concluded, therefore, that reduction of the number of fully equipped mitochondria indeed leads to impairment of an energy-requiring process such as cell division.

Administration of oxytetracycline by means of continuous infusion made it possible to inhibit mitochondrial protein synthesis in rats during long periods and to study the consequences thereof not only for proliferating tumor cells, but also for normal, resting cells [7,8]. Continuous infusion allows also the maintenance of the desired low oxytetracycline serum levels, causing inhibition of mitochondrial protein synthesis only [7]. Temporary high serum levels, which can lead to nonspecific side effects, are avoided in this way [9]. This prolonged and controlled treatment opens therefore the possibility of defining the level to which the oxidative phosphorylation capacity in liver cells can be reduced before serious consequences on or during liver regeneration are the result. The results of such studies are the subject of the present paper.

Materials and Methods

Animals and reagents. Male Wistar rats, weighing 250–300 g, were used in all experiments. Oxytetracycline in its hydrochloride form (Vendarcin) was obtained from Gist-Brocades N.V. (Delft, The Netherlands). All other chemicals used were of analytical grade.

Partial hepatectomy. Rats were partially hepatectomized according to the method of Higgins and Anderson [10], under ether anesthesia. The operations were always carried out at the same time of day.

Administration of oxytetracycline. Oxytetracycline, dissolved in infusion solution, was administered by means of continuous intravenous infusion via the jugular vein as described previously [5,7]. The infusion solution consisted of 0.15 M NaCl, 50 U/l heparin to prevent clotting and was adjusted to pH 2.5 with HCl. Control animals were similarly infused, with infusion solution only.

Analytical methods. The oxytetracycline content of serum and liver homogenates was determined as described before [11]. Blood was hereto aspired from the vena cava inferior at the end of each experiment and serum obtained by centrifugation of the blood samples. 10% liver homogenates were made in 0.15 M NaCl.

The protein content of the liver was determined with a modification [12] of the method of Lowry et al. [21], the DNA content fluorometrically [13].

Cytochrome *c* oxidase activity in the liver was assayed spectrophotometrically at 20°C [14]. The activity was calculated per min and expressed as the first-order reaction rate constant. The specific activity was expressed per mg protein.

Results

Maintenance of constant oxytetracycline serum levels

As stated in the Introduction, continuous infusion should assure the maintenance of desired serum levels of the drug infused. As shown in a previous study [7], a given dose of oxytetracycline, administered continuously for 1 week, leads to constant serum levels. In experiments requiring oxytetracycline administration for periods of more than 1 week (Ref. 6, and this study) it became clear that after 3 weeks of constant treatment at a dosage of 20 mg/kg per day, initially high enough to inhibit mitochondrial protein synthesis in all tissues investigated [7], no further effects are found. The explanation for this phenomenon can be found in Fig. 1, which shows clearly, that after 1 week of treatment with a given amount of oxytetracycline, oxytetracycline serum levels become gradually lower.

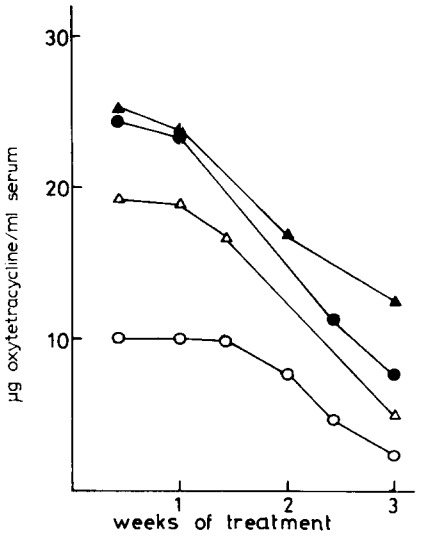

Fig. 1. Serum levels of oxytetracycline after various periods of treatment with different amounts of the antibiotic. (○——○) 20, (△——△) 40, (●——●) 60, (▲——▲) 80 mg/kg per day. Each point represents the mean value found in three animals. Individual values deviated maximally 1.2 µg/ml from the mean.

The cause of the declining serum levels was not further investigated. We assume, however, that after a certain period of treatment with oxytetracycline somehow a mechanism for the removal of oxytetracycline at an ever-increasing rate becomes induced. On treatment with high doses accelerated removal of oxytetracycline occurs even without pretreatment, because the linear relationship between dose and serum level is lost at doses of 40 mg/kg per day and higher (Fig. 1 and Ref. 7).

Oxytetracycline is generally thought to be metabolically inert [15] and to be excreted by the kidneys. Therefore, the liver does not play a major role in the biotransformation of oxytetracycline under normal conditions. Serum levels found after lengthy periods of treatment were similar in oxytetracycline-treated controls and partially hepatectomized, oxytetracycline-treated rats. The removal of 70% of the liver, therefore, does not influence the rate of oxytetracycline excretion. The mechanisms supposed to be operable are thus also not likely to be located in the liver.

The data shown in Fig. 1 enabled us to design regimes for maintaining oxytetracycline serum concentrations at a level inhibiting mitochondrial protein synthesis specifically, also for periods lasting more than 1 week. The administration of 80 mg/kg per day during the whole experimental period, or the weekly doubling of an initial dose of 20 mg/kg per day did not lead to different observations as far as the specific inhibition of mitochondrial protein synthesis was concerned. Both regimes have been used in this study.

Effect of oxytetracycline on liver regeneration

During partial hepatectomy 70% of the liver is removed. The liver remnant contains therefore 30% of the original cytochrome *c* oxidase activity and of the original amounts of DNA and protein. In

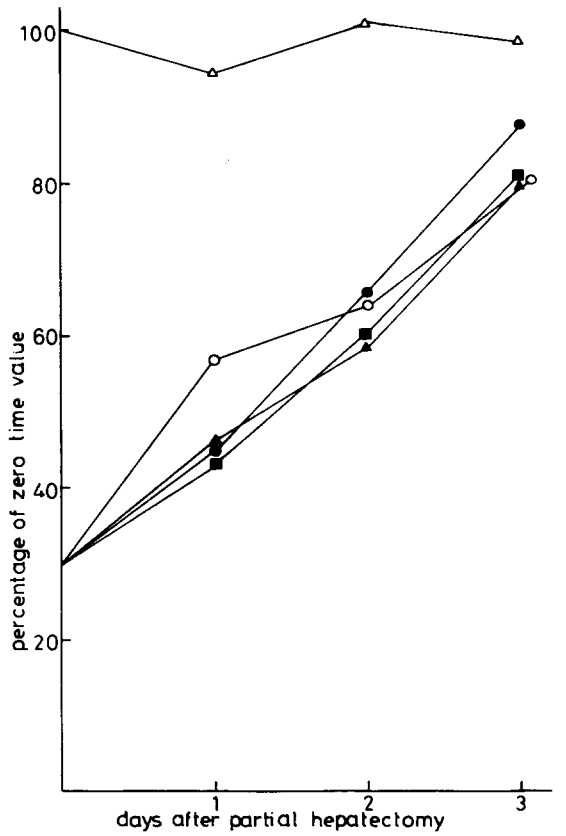

Fig. 2. Liver weight (●——●), DNA content (○——○), protein content (▲——▲) and specific (△——△) and total (■——■) cytochrome *c* oxidase activity of regenerating liver of control animals. 100% is the value immediately before hepatectomy. Values are given as the means. For purposes of clarity, the S.E. is not given in the figure; S.E. values varied between 1 and 4.5%. Each point is the average of 3 (1 day), 6 (2 days) or 12 (3 days) experiments.

control animals the increases in wet weight, amount of protein and DNA and cytochrome *c* oxidase activity follow a similar pattern. As shown in Fig. 2, the parameters studied show doubling at the second day and an increase to about 80% of the zero time value at the third day after partial hepatectomy. It is also clear from Fig. 2 that the specific cytochrome *c* oxidase activity remains at its original 100% level. Since this enzyme is composed of polypeptides of cytoplasmic and of mitochondrial translational origin, this indicates that the regeneration is based on a coordinate activity of the two genetic systems and coincides with total protein regeneration.

If mitochondrial protein synthesis is inhibited by oxytetracycline, it is expected that the regeneration of the cytochrome *c* oxidase activity is reduced. If inhibition of mitochondrial protein synthesis had consequences for normal liver regeneration, the course of DNA, protein or wet weight regeneration curves should also be affected. The same should hold for conditions leading to oxytetracycline concentrations impairing cytoplasmic protein synthesis directly.

Figs. 3 and 4 show the results of inhibition of mitochondrial protein synthesis on liver regeneration. The results given in Fig. 3 were obtained at oxytetracycline serum levels varying between 4 and 21 μg/ml. These serum levels appear to be

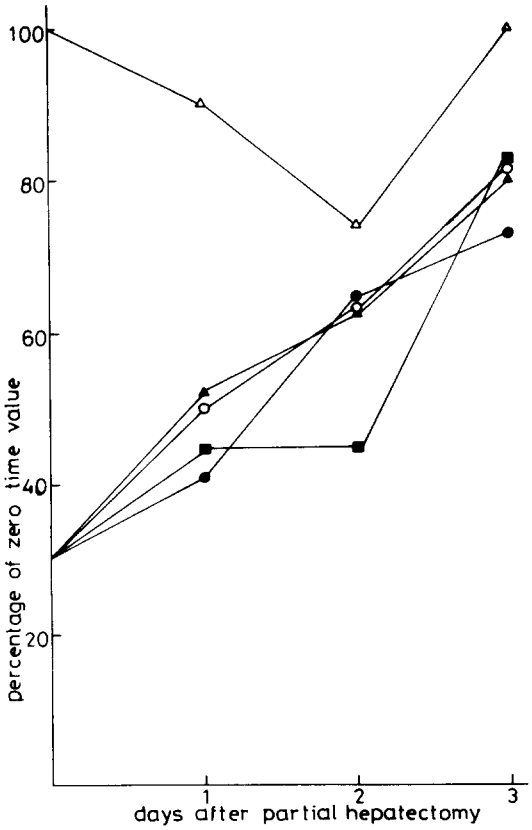

Fig. 3. Liver weight (●———●), DNA content (○———○), protein content (▲———▲) and specific (△———△) and total (■———■) cytochrome *c* oxidase activity of regenerating liver of oxytetracycline-treated animals. Serum levels of oxytetracycline ranged in these experiments from 5 to 20 μg/ml. The number of experiments was 2 (1 day), 6 (2 days) and 4 (3 days); the S.E. 1–5%. Further information can be found in the legend to Fig. 2.

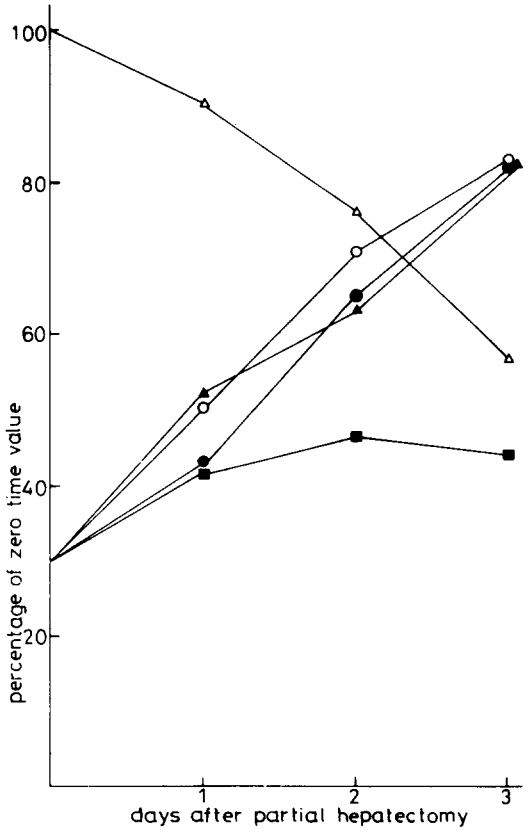

Fig. 4. Liver weight (●———●), DNA content (○———○), protein content (▲———▲) and specific (△———△) and total (■———■) cytochrome *c* oxidase activity of regenerating liver of oxytetracycline-treated animals. Serum levels of oxytetracycline ranged from 21 to 25 μg/ml in these experiments. The number of experiments was 2 (1 day), 6 (2 days) and 5 (3 days); the S.E. 1–5%.

high enough to inhibit mitochondrial protein synthesis during the first and second day after partial hepatectomy, since the formation of cytochrome *c* oxidase is retarded. No differences compared to control experiments are found regarding the increase in amount of protein, DNA or wet weight. The specific cytochrome *c* oxidase activity declines gradually, therefore, during the first 2 days after partial hepatectomy. On the third day, however, normal values for all five parameters are found. This implies that mitochondrial protein synthesis is no longer impaired by oxytetracycline, though the serum level remains constant. The inhibition is, moreover, reversible. It shows further, that between 2 and 3 days after partial hepatectomy, the rate of mitochondrial protein synthesis becomes even enhanced under these circumstances.

The observed lack of continuous inhibition of mitochondrial protein synthesis in regenerating liver at the serum levels of oxytetracycline, which are high enough to impair this process in normal liver, is based on differences in oxytetracycline concentrations in normal and regenerating liver. In tissue-distribution studies [7], the liver was always found to contain at least twice as much oxytetracycline per g wet weight as per ml serum. In regenerating liver the same ratio was found at the first day after partial hepatectomy, thereafter, however the oxytetracycline content of the liver declined rapidly. A reduced or changed pattern of blood supply in regenerating liver, leading to lowered oxytetracycline concentrations in the liver, seems the most likely explanation. Due to the growth of the remaining 30% of the liver during regeneration the oxytetracycline already present will be diluted, while the supply from the blood is not yet sufficient. The administration of higher doses of oxytetracycline during liver regeneration should overcome this difficulty if the tissue-to-serum oxytetracycline ratio remains constant.

In the experiments shown in Fig. 4, the oxytetracycline serum levels were raised to values between 21 and 25 μg/ml. At these levels, the cytochrome *c* oxidase activity indeed remains constant during regeneration, implying that the oxytetracycline tissue level is high enough to cause inhibition of mitochondrial protein synthesis under these conditions. The DNA, protein and wet weight curves again have a similar shape to that obtained using control animals. The specific cytochrome *c* oxidase activity on the third day after partial hepatectomy is now further reduced. Therefore, diminution of the specific activity to about 50% does not lead, as shown before [2], to impairment of the regeneration of liver wet weight, or of the amount of DNA or protein.

Effect of oxytetracycline on liver regeneration after pretreatment

From the experiments presented above it follows that the reserve of respiratory capacity in the liver is at least 50%. To obtain a threshold value, below which an energy-demanding process like liver regeneration becomes impaired, the functional capacity of the mitochondria must already be lowered before partial hepatectomy. To achieve this, rats were pretreated with oxytetracycline for various periods. It is known that the mitochondrial half-life in liver is 9–10 days. Administration of oxytetracycline in amounts sufficiently high for specific inhibition of mitochondrial protein synthesis will thus reduce the ATP-generating capacity by 50% every 9 days.

In Fig. 5, liver regeneration, expressed as the percentage of the DNA and protein amount of the zero time value at the third day after partial hepatectomy, is plotted against the duration of the pretreatment period. It can be seen that 18 days of pretreatment, causing reduction of the specific cytochrome *c* oxidase activity just before partial hepatectomy to about 25% of the normal value, still does not cause impaired liver regeneration. A pretreatment period of 22 days, however, significantly inhibits regeneration. Treatment for 22 days reduces the specific cytochrome *c* oxidase activity before hepatectomy to 20% (Table I). This reduction alone is still not enough to cause a lack of energy, because if mitochondrial protein synthesis is not also inhibited after the second day of liver regeneration (Fig. 5, open symbols) no adverse effects are found.

If the oxytetracycline serum level is raised to inhibit mitochondrial protein synthesis also during the third day of liver regeneration, the DNA and protein amount found on the third day equal those found on the second day after partial hepatectomy. This indicates that the specific cytochrome *c*

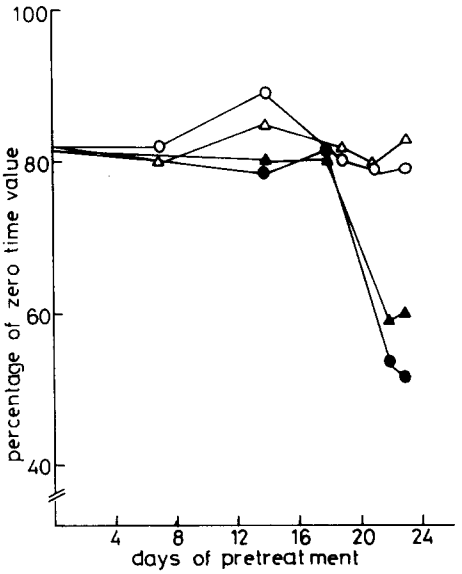

Fig. 5. Protein (△———△; ▲———▲) and DNA content (○———○; ●———●) of regenerating liver of rats, pretreated for varying periods with oxytetracycline as measured on the third day after partial hepatectomy. During the period of pretreatment oxytetracycline serum levels were kept between 10 and 15 μg/ml, whereas during liver regeneration oxytetracycline serum levels varied between 4 and 20 (△———△; ○———○) or 21 and 25 μg/ml (▲———▲; ●———●). Each point is the average of 3–7 experiments, the S.E. was 1–4%. 100% is the value found immediately before partial hepatectomy.

TABLE I

SPECIFIC CYTOCHROME *c* OXIDASE ACTIVITIES IN LIVER HOMOGENATES, BEFORE AND AFTER PARTIAL HEPATECTOMY, OF RATS TREATED IN VARIOUS WAYS WITH OXYTETRACYCLINE

Each value is the mean of determinations in 7–15 rats. The S.D. varied between 1 and 1.8%. Between the brackets the percentage of the control value (column 1) or of the zero time value (column 2) is given.

Oxytetracycline serum level during liver regeneration	Days of pretreatment	Specific cytochrome *c* oxidase activity	
		Before partial hepatectomy	After partial hepatectomy (3rd day)
4–20 μg/ml	0	27.1 (100)	27.1 (100)
	7	16.2 (60)	16.7 (103)
	14	9.7 (36)	11.5 (119)
	19	6.9 (26)	8.5 (123)
	21	5.8 (21)	7.7 (133)
	23	5.0 (19)	6.8 (136)
21–25 μg/ml	0	27.9 (100)	15.1 (56)
	15	9.1 (34)	4.9 (54)
	18	7.3 (27)	4.1 (56)
	22	5.4 (20)	3.7 (69)
	23	5.0 (19)	3.6 (72)

oxidase activity, and thus the respiratory capacity, can be halved once more after 22 days of pretreatment before inhibitory effects are found. A specific cytochrome *c* oxidase activity of about 10% of the normal value is therefore the critical limit.

This value can also be calculated from Table I. In this table the specific cytochrome *c* oxidase activities found in liver after various periods of pretreatment are shown. The specific cytochrome *c* oxidase activities measured at the third day of liver regeneration at various oxytetracycline serum levels are also given.

The gradual decline of the specific activity of cytochrome *c* oxidase found if the period of pretreatment lasts longer confirms first of all the estimation generally made for mitochondrial half-life in liver. Secondly, it can be calculated from Figs. 3 and 4 and Table I that the specific cytochrome *c* oxidase activity in liver can be reduced to 14% of its normal value before the consequences of diminished ATP-generating capacity are observed. The latter is the value expected if mitochondrial protein synthesis is still inhibited on the second day after partial hepatectomy after a pretreatment period of 23 days. If, as is the case at oxytetracycline serum levels between 21 and 25 μg/ml, mitochondrial protein synthesis is inhibited further, inhibition of cytoplasmic protein synthesis and thus of liver regeneration is the result. The specific cytochrome *c* oxidase activity, therefore, remains also at 70% of its zero time value under these circumstances, because this parameter is expressed on a total protein base.

Another conclusion from the data of Table I is that the longer the pretreatment period has lasted, the faster cytochrome *c* oxidase is newly formed as soon as the block on mitochondrial protein synthesis is removed. The enhancement of mitochondrial protein synthesis, also found in the presence of 4–20 μg oxytetracycline/ml serum (Fig. 3) after the second day of liver regeneration

without pretreatment, seems thus related to the degree to which mitochondriogenesis has been reduced.

Discussion

The results of this study confirm once more that mitochondrial protein synthesis in the intact animal can be blocked fairly completely by the tetracyclines. To observe such effects of these common antibiotics, maintenance of adequate serum levels is a prerequisite. Administration of the tetracyclines via methods other than continuous infusion may lead to serum levels which are alternatingly too high, causing nonspecific side effects, and too low to inhibit even mitochondrial protein synthesis. In the latter case, mitochondria can respond, as shown in this study, with an enhanced rate of protein synthesis. Possible effects of eukaryotic cells will then be reversed and thus not be noticed.

At the other hand, one has also to reckon with an enhanced rate of tetracycline removal after prolonged treatment. If such a phenomenon is also operative in humans, it may contribute to the relatively few side effects described after antibiotic treatment for several months [16,17].

By carefully controlling the serum (and tissue) levels of the tetracyclines, conditions can be chosen which make it possible to study the effects of prolonged continuous inhibition of mitochondrial protein synthesis. In previous studies [5,6,18] it was already reported that cell division arrest may be a result of the impairment of the formation of fully equipped mitochondria. However, from these and other studies it appears also that large reserve capacities for ATP generation exist in various kinds of cells.

From the results obtained in the present study, it can be concluded that in regenerating liver cells the number of fully equipped mitochondria can be reduced to 10–14% before it leads to energetic or metabolic consequences, resulting in impairment of liver regeneration. A reserve capacity for ATP generation of about 85% in these cells is therefore likely.

The possibility cannot be excluded that in normal, resting liver cells an even greater reserve capacity is present. In regenerating liver an enhanced demand on oxidative phosphorylation [19] and a growing metabolic load upon the liver remnant are exerted, whereas simultaneously the liver cells have to generate much ATP for cell division and growth. These processes will undoubtedly call on the reserves for oxidative phosphorylation.

The results indicate that in normal liver cells the number of fully equipped respiratory chains must be reduced to about 90% before serious side effects can be expected. This large reserve capacity for ATP generation might be related to the well known high mitochondrial content of liver cells. We have hypothesized previously that this may be a more general phenomenon [5]. This view is supported by comparison of the results of the present investigation with those obtained in studies on the effect of prolonged inhibition of mitochondrial protein synthesis in Zajdela tumor cells. Zajdela hepatoma cells have arisen from rat liver cells by treatment with a carcinogen and are rather dedifferentiated. Dedifferentiated tumor cells generally contain less mitochondrial enzymes than their normal adult counterparts. They contain therefore less mitochondria, which may explain in part the relatively large contribution of glycolysis to ATP generation [20].

Using cytochrome *c* oxidase activity as a marker for functional mitochondria, we found that Zajdela tumor cells contain on a cell basis about 10% [5] of the amount of mitochondria in normal liver cells. Proliferation arrest of Zajdela tumor cells was found when the cytochrome *c* oxidase activity was reduced to about 40% of the control value. The reserve capacity in these liver-derived cells is, therefore, only 60%. The experiments offer a clear example, therefore, of a different reserve capacity for oxidative phosphorylation in two related cell types. The different behavior of these two cell types as far as their proliferation activity is concerned can be explained by their dissimilar mitochondrial contents on a cell basis. The consequences of inhibition of mitochondrial protein synthesis thus become apparent much earlier in the cells with the lowest reserve capacity.

The conclusion drawn above for liver cells supports our hypothesis but may have a greater impact. It implies, for instance, that the accelerated rate of glycolysis often found in dedifferentiated tumor cells is indeed a sheer necessity based on

reduced oxidative ATP-generating capacity. The results further support our assumption [5,6] that mitochondria are a possible target for anticancer chemotherapy, because of the reduced oxidative phosphorylating capacity in dedifferentiated tumor cells on the one hand, and the higher turnover rate of tumor cells in general on the other.

Acknowledgements

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