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ORIGINAL ARTICLE

Adriamycin cytotoxicity may stimulate growth of hepatocellular tumours in an experimental model for adjuvant systemic chemotherapy in liver transplantation

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Summary

Adjuvant treatment with adriamycin has been suggested to improve results after liver transplantation for hepatocellular cancer. Here we have applied an animal model for evaluation of treatment with adriamycin and/or cyclosporine A on liver tumour growth. Three chemically induced rat liver tumours with various degree of differentiation were transferred to the spleens of syngenic rats. Each recipient group was divided into four subgroups, treated with adriamycin and/or cyclosporine A or none of the drugs. When the tumour was well differentiated no proliferation was found in any of the subgroups. When the tumour exhibited a more pronounced dysplasia, adriamycin stimulated tumour growth. This effect was further increased by cyclosporine. In the animals transplanted with the most aggressive tumour, adriamycin inhibited tumour growth. When given together with cyclosporine this inhibition was counteracted. These data suggest that adriamycin, especially when given together with cyclosporine, may have a stimulatory effect on liver tumour cell growth.

Introduction

Hepatocellular carcinoma (HCC) has a uniformly low sensitivity to chemotherapy both *in vivo* [1] and *in vitro* [2]. The best results have been reported with intra-arterial administration, with or without concomitant embolization (TACE), where tumour response can be achieved in 25–64% of cases [3,4]. The most commonly used drugs are combinations with adriamycin (doxorubicin)/epirubicin, 5-fluorouracil (5 FU), mitomycin C and *cis*-platinum. Most randomized trials have failed to show any survival benefit of TACE [5,6] but data are conflicting and some short-term positive effects have recently been reported [4]. Trials with systemic chemotherapy for advanced HCC have so far been disappointing, having shown no improved survival despite initial tumour responses [7].

For patients with non-resectable intrahepatic HCC, orthotopic liver transplantation (OLT) offers the best chance of palliation and, in some patients, cure from the disease [8]. However, because of a high recurrence rate, long-term results are inferior to results with OLT for other established indications. Adjuvant treatment with chemotherapy following OLT may have a place in the treatment of HCC but data are so far collected only for small series of patients and no randomized controlled study has yet been conducted [9]. It has been suggested that systemic low-dose adriamycin if given as a neo-adjuvant treatment during the first post-transplant year would have a positive effect on survival after OLT [10]. A randomized, multi-centre trial testing this hypothesis is currently underway (Nordic Liver Transplantation Group).

From an experimental point of view, however, there are some concerns with such a protocol. It is well known

that liver tumour cells may develop resistance against the cytotoxic effect of adriamycin [11,12] and it has been proposed that this resistance arises from increased scavenging of free radicals because of elevated levels of antioxidants such as glutathione (GSH) and α-tocopherol (vitamin E) [13]. Moreover, adriamycin may also retard liver cell regeneration after surgery and therefore necessitate a prolonged period of growth stimulation with release of several growth factors in order to restore the original volume of the liver [14]. This growth stimulatory effect may be hazardous in a patient with possible remaining liver tumour cells as these cells may have a less developed response to the mito-inhibition exerted by adriamycin compared with normal liver cells [15]. Another factor to consider is that immunosuppression is routinely given after OLT. Recently mTOR inhibitors have been suggested to have some anti-tumoral effects *in vitro* and *in vivo* [16,17]. Partly this effect has been explained by the direct inhibition of vascular endothelial cell growth factor production and decreased neo-angiogenesis. The effect of mTOR inhibitors on tumour recurrence after OLT caused by HCC is, however, still unexplored and the use of these inhibitors in the clinical practice is limited. Although most of the other immunosuppressive drugs *per se* may promote tumour cell proliferation [18,19], little is known about the clinical interaction between the different immunosuppressive drugs and adriamycin. Cyclosporine A has been shown to enhance the effect of cytotoxic drugs in a variety of cells [20]; however, when applied *in vivo* the outcome has been complicated by non-specific toxicity resulting in severe side-effects [21]. The effect on the antioxidant regenerating substances in the liver has not yet been studied.

The present study, in which we transplanted chemically induced HCC into the spleens of syngenic rats, was designed to provide an animal model for treatment of HCC in patients using a combination of OLT, adjuvant chemotherapy with adriamycin and immunosuppression with a low dose of cyclosporine A.

Materials and Methods

Chemicals

The 2-acetylaminofluorene (2-AAF)-containing diet (0.05%, w/w) was prepared by Altromine (Lage, Germany). Adriamycin® was supplied by Pharmacia (Stockholm, Sweden) and cyclosporine A (Sandimmune®) by Sandoz Pharma Ltd (Basle, Switzerland). Micro-osmotic pumps were obtained from Alza Corporation and the immunohistochemical bromodeoxyuridine (BrdU, Palo Alto, CA, USA)-staining kit, from Dakopatts AB (Stockholm, Sweden), while glutathione disulphide (GSSG) reductase was obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were purchased from Sigma (Stockholm, Sweden).

Animal experiments and diets

Inbred male Wistar Kyoto rats were obtained from Møllegaards Breeding Centre (Ejby, Denmark) at an age of 4–5 weeks and a weight of about 110 g. The donor rats ($n = 3$) were maintained under standard conditions on a basal diet until initiation of the feeding protocol, which consisted of intermittent administration of a 2-AAF-containing diet for 22 weeks. After a total of 33–42 weeks, the donor rats were killed and hepatomas suitable for transplantation were harvested (Fig. 1a). A slice of the tumour was used for histological classification and three different kinds of tumours were selected. Tumour type I exhibited a low to moderate grade of nuclear dysplasia (Fig. 2a) while tumour type II consisted of cells with moderate to severe nuclear polymorphism (Fig. 2b). Tumour type III was an HCC of a mixed trabecular and acinar type with severe dysplasia and revealed also abundant neovascularization (Fig. 2c). The remainders of the three tumours were minced and placed in isotonic saline at room temperature.

The recipient rats ($n = 135$) were divided into three groups, each receiving one of the three different donor tumour types at an age of 7–8 weeks. The animals were maintained under standard conditions and on the basal diet throughout the entire experiment. The initial step in the tumour transfer procedure was a partial two-thirds hepatectomy (in order to create a regenerative burst and a tumour growth promoting environment) of the recipient, after which 20 µl of the suspension of minced tumour tissue was injected into the spleen using a 1.6 mm syringe. A small pad of Spongostan was pressed against the spleen in order to stop bleeding and prevent loss of tumour cells by leakage.

Each tumour type group consisting of 45 rats was subdivided into four treatment groups. Subgroup A (10 rats) was treated intravenously (i.v.) with adriamycin [0.5 mg/kg body weight (BW)] once a week. Subgroup B (10 rats) was treated intraperitoneally (i.p.) with cyclosporine A (2.5 mg/kg BW) twice weekly. Subgroup C (10 rats) was treated with both adriamycin (in the same manner as group A) and cyclosporine (in the same manner as group B). Subgroup D (15 rats) consisted of untreated controls. Treatment was carried out for 22 weeks (tumour type I). For tumour types II and III, the treatment was maintained for 17 and 5 weeks respectively. The doses of cyclosporine and adriamycin were calculated from previous experiences with this rat strain in order reach low maintenance concentrations (cyclosporine A) but to avoid non-specific organ toxicity under long-term treatment in

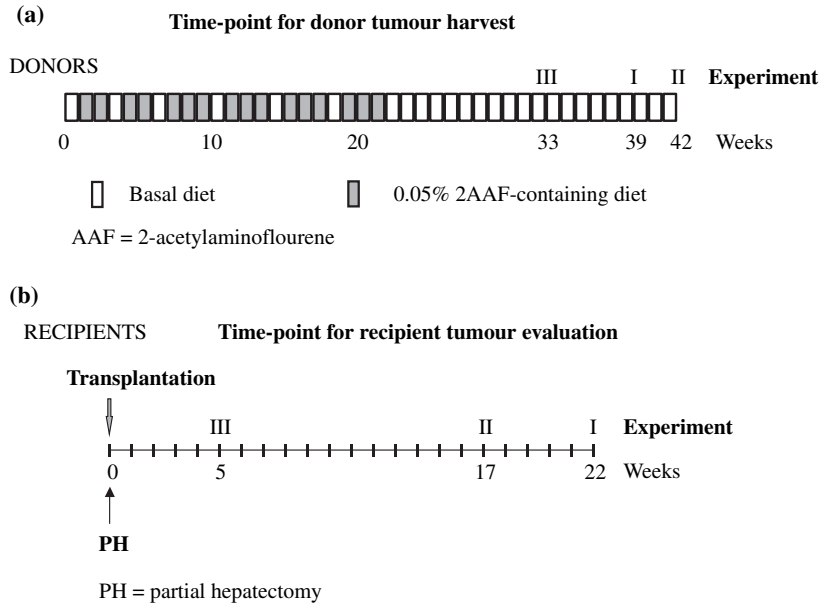


Figure 1 Schematic presentation of (a) the intermittent 2-acetylaminofluorene (2-AAF) feeding schedule which donor rats were subjected to, and (b) the protocol for recipient animals. Tumours for transplantation were harvested 33–42 weeks after initiation of the feeding protocol.

the combination therapy group (Rissler, Söderdahl and Eriksson). The i.p. route [22] for the long-term administration of cyclosporine A was chosen for the sake of convenience while adriamycin had to be given i.v. in order to avoid severe peritonitis.

The rats were killed when the tumour mass became palpable and/or when other signs of tumour growth or toxicity, such as weight loss, appeared in several of the animals (Fig. 1b). All surviving rats in each tumour group were killed at the same time. Tumour mass in the spleen was estimated by the wet weight of the entire organ (control values, i.e. without tumours, were consistently 0.4–0.5 g), which corresponded well to the size of the visible tumour.

Three days prior to the termination of each experiment, osmotic mini-pumps containing BrdU were implanted subcutaneously (s.c.) in all animals. These micro-osmotic pumps were filled with 0.4 M BrdU dissolved in 0.5 M NaOH and activated by 4-h incubation in isotonic saline at 37 °C. Pieces of the livers were fixed in neutral buffered 4% formaldehyde and embedded in paraffin. Sections 4 μm in thickness were stained immunohistochemically for BrdU, according to the supplier's instructions. A BrdU-labelling index (LI) was determined by randomly examining 800–1 000 cells in each liver lobe under high magnification.

Preparation of liver homogenates and cytosol

Liver samples were harvested and prepared in 50 mM sodium phosphate buffer, pH 7.0, by use of a homogenizer equipped with knives (Polytrone® PT 1200; Lucerne, Switzerland). For determination of the GSH concentration, the homogenates were prepared with 5% metaphosphoric acid w/v and stored under N_2. For enzyme measurements and Western blot analysis, cytosol was obtained by ultracentrifugation (105 000 *g*, 60 min, 4 °C) of the homogenates. Protein was determined according to a method described by Lowry *et al.* [23].

Analysis of ubiquinol, ubiquinone and vitamin E

A reversed-phase high-performance liquid chromatography (RP-HPLC) system connected to both ultraviolet and electrochemical detectors was used for analysis of ubiquinol, ubiquinone and vitamin E. As internal standards, δ-tocopherol (vitamin E) and ubiquinone-6 were utilized [24].

Glutathione levels

Reduced GSH was measured using Bioxytech® GSH-400™ assay (OXIS International Inc., Portland, OR, USA). After centrifugation (3000 *g*, 10 min, +4 °C) GSH levels were analysed spectrophotometrically at 400 nm. The concentrations were calculated using a standard curve.

Glutathione reductase activity

Glutathione reductase activity was analysed essentially as described by Carlberg and Mannervik [25]. Briefly, the reaction mixture contained 50 mM potassium phosphate buffer, pH 7.6, 1 mM ethylenediamine tetra-acetic acid (EDTA) and 0.2 mM dihydro-nicotinamide-adenine-

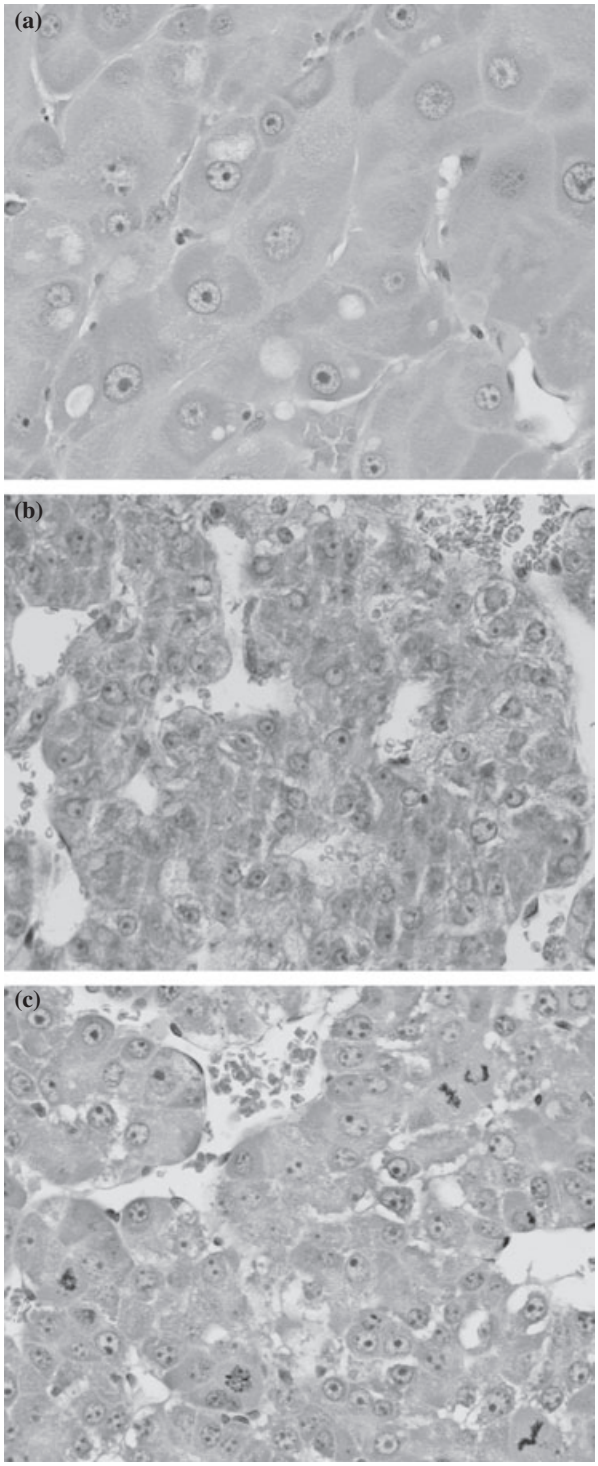


Figure 2 (a–c) Histology of the three different donor tumours used for transplantation.

dinucleotide phosphate (NADPH). The oxidation of the NADPH was followed spectrophotometrically (Shimadzu PC2501; Shimadzu, Kyoto, Japan) at 340 nm, 25 °C, after addition of the cytosol fraction and GSSG to the reaction mixture. The activities were calculated using a molar extension coefficient of 6.22.

Statistical analysis

All statistical analyses were performed using Student's *t*-test. Data are given as mean ± standard deviation (SD).

The experimental protocol was approved by the Southern Stockholm Committee for Ethical Review of Animal Experimentation and the 'Principles of laboratory animal care' (NIH publication No. 86-23, revised 1985) were followed.

Results

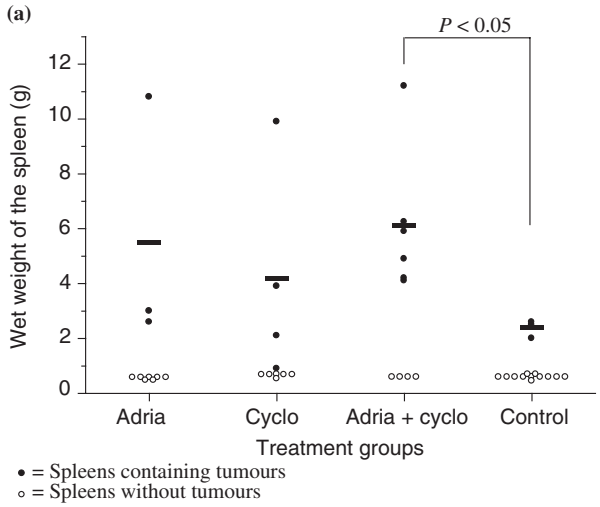
Tumour growth

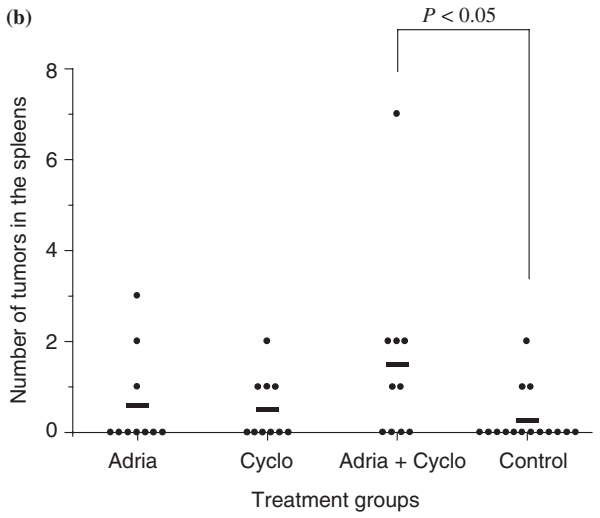
The rats transplanted with the tumour exhibiting a low grade of dysplasia (tumour type I) were treated until weight loss was observed in the subgroups receiving adriamycin (22 weeks). In this experiment no tumour growth was noted in any of the animals, although a few small groups of tumour cells at the site of injection were seen in some of the spleens. The total volume of these tumours was much smaller than that of the pieces originally transplanted.

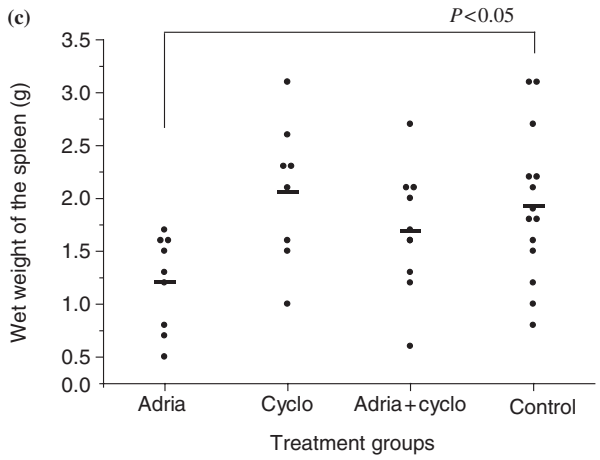
In rats transplanted with tumour pieces demonstrating a moderate grade of dysplasia (tumour type II) established tumours could be palpated after 15–16 weeks in several of the animals and the experiment was terminated after 17 weeks. Examination of the spleens revealed tumour growth in 35% of the animals.

In the subgroup of animals treated with adriamycin alone (subgroup A) tumour mass was increased, however not significantly, compared with the control group. This effect was further augmented and reached statistical significance when the animals received combined treatment with adriamycin and cyclosporine (subgroup C). Cyclosporine alone had a small but insignificant stimulatory effect on tumour growth (Fig. 3a). Adriamycin also influenced tumour establishment, as reflected by the fact that adriamycin and cyclosporine together, but not cyclosporine alone, significantly increased the number of splenic tumours (Fig. 3b).

In the third group transplanted with tumour demonstrating severe dysplasia and neovascularization three rats died prior to the appearance of palpable tumour masses – one in the subgroup receiving adriamycin and two in the subgroup treated with cyclosporine. The cause of death was massive intra-abdominal bleeding. In two rats this bleeding had destroyed the splenic parenchyma, making determination of organ weight and tumour growth impossible. When this experiment was terminated after







5 weeks, extensive tumour growth was observed in the spleen of all rats. With transplantation of this tumour, adriamycin alone significantly inhibited tumour growth but when co-administered with cyclosporine this inhibition was counteracted and not significant. Cyclosporine alone was without effect on tumour growth (Fig. 3c).

Table 1. Mean effects (±SD) of the various treatments on the bromodeoxyuridine (BrdU)-labelling index (LI) of normal hepatocytes and on relative liver weight in rats with tumour types I and II. Student's *t*-test was used to compare the treated groups with the control group.

Treatment	LI	Relative liver weight
Adriamycin	1.6 ± 1.9***	3.3 ± 0.2
Cyclosporine	0.3 ± 0.3	2.6 ± 0.1
Adriamycin + cyclosporine	1.4 ± 0.9***	3.2 ± 0.2
Control (untreated)	0.3 ± 0.3	2.5 ± 0.1

***$P < 0.001$.

Liver proliferation

Analysis of the rats belonging to the two tumour groups with a relatively long period of treatment (tumour types I and II) revealed that proliferation of non-tumorous hepatocytes, expressed as the BrdU-LI, was increased by adriamycin or the combination of adriamycin and cyclosporine. Cyclosporine alone did not alter the LI. The same pattern of growth stimulation was reflected in relative liver weights, so that adriamycin either alone or in combination with cyclosporine significantly increased liver weight while cyclosporine by itself had no effect (Table 1).

Lipid-soluble antioxidant levels and gluthatione reductase activity in the liver

The levels of the lipid-soluble antioxidants ubiquinol (reduced Q9), ubiquinone (oxidized Q9) and vitamin E

Figure 3 (a) Wet weight of spleen tissue (i.e. splenic tumour mass) in animals transplanted with tumour type II with or without drug treatment. The mass of non-tumorous splenic tissue was 0.4–0.5 g in all groups. Each circle represents an individual rat. Filled circles represent spleens containing tumours. Unfilled circles represent spleens in which no tumours could be found. Bars represent average values for spleens containing tumours. Statistical analysis employing Student's *t*-test was used to compare treated groups with the control group. (b) Number of tumours in the spleen of animals transplanted with tumour type II with or without drug treatment. Each circle represents an individual rat. The bar represents average values. Student's *t*-test was used to compare treated groups with the control group. (c) Wet weight of spleen tissue in animals transplanted with tumour type III with or without drug treatment. Rats that had died or that exhibited haemorrhagic spleen were excluded. Each circle represents an individual rat. The bars represent average values. Student's *t*-test was used to compare treated groups with the control group.

in tumour-free liver tissue from the different subgroups were analysed simultaneously by RP-HPLC. No differences in antioxidant levels were found between any of the groups after 5 weeks of treatment. When treatment was extended to 17 weeks a significant rise in the lipid-soluble antioxidant level was found only in the group of rats that had received the combination of adriamycin and cyclosporine. After 22 weeks, treatment with adriamycin alone or adriamycin in combination with cyclosporine resulted in a two to threefold increase in lipid-soluble antioxidants. After 17 and 22 weeks of treatment the fraction of active, reduced Q9 showed a tendency to decrease in all treated groups, but most markedly in animals receiving adriamycin alone. The difference did not reach statistical significance. The total amount of ubiquinol (and ubiquinone) was, however, increased (Fig. 4a–d).

The levels of GSH after 5 weeks of treatment were found to be equal in all groups. There were no differences between controls and the group treated with cyclosporine A alone at any time point. However, after 17 weeks of treatment with adriamycin and with a combination of cyclosporine and adriamycin there was a 30% and 60%

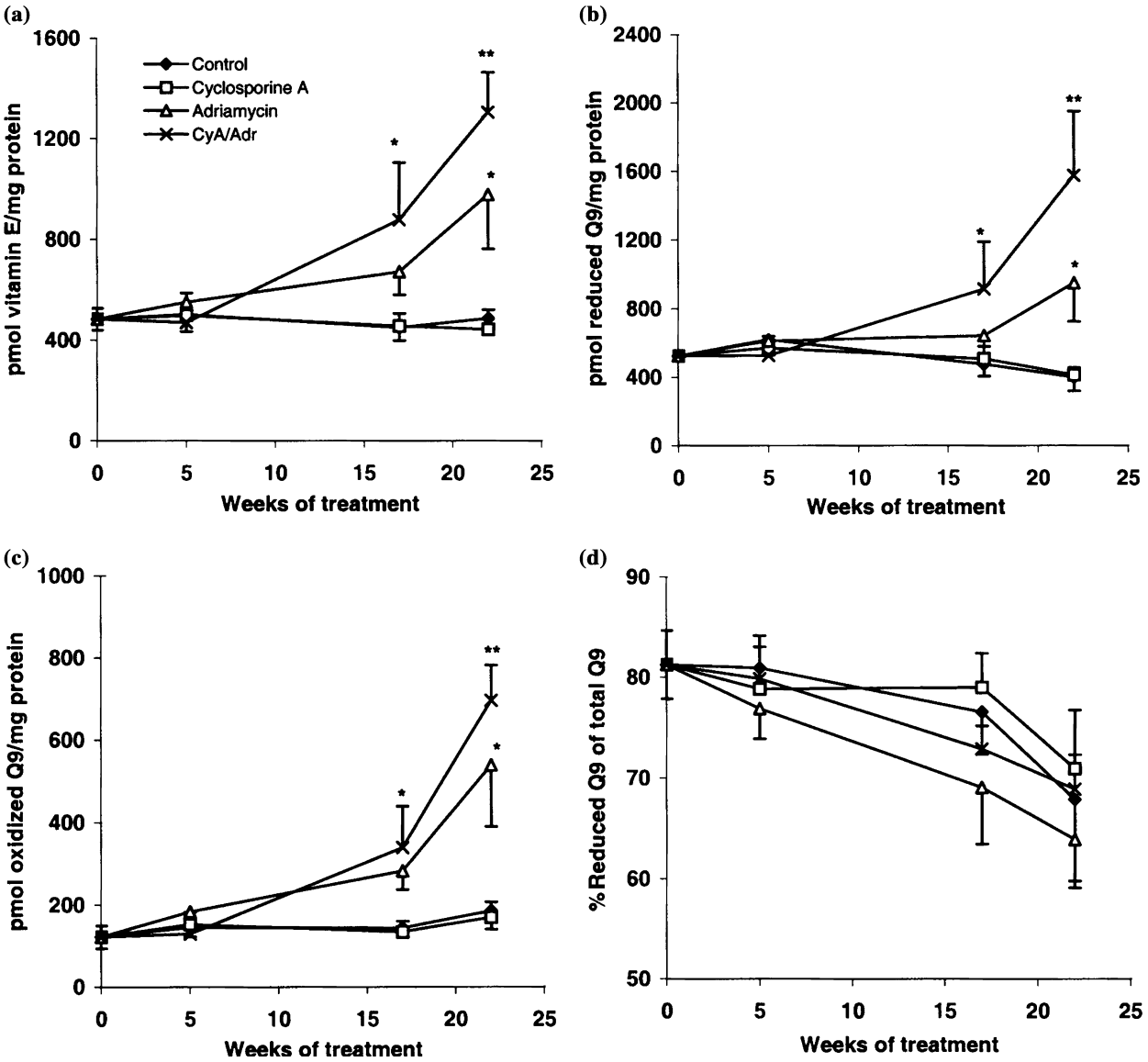


Figure 4 Levels of (a) vitamin E; (b) reduced Q9; and (c) oxidized Q9. (d) The percentage of total Q9 which was reduced before and after treatment with cyclosporine A and/or adriamycin for 5, 17 or 22 weeks. Control rats (♦); rats treated with cyclosporine A (□); adriamycin (△); and a combination of adriamycin and cyclosporine (x). Where indicated, the results are significantly different from those of controls at the levels of $*P < 0.05$ and $**P < 0.01$, as determined using Student's *t*-test.

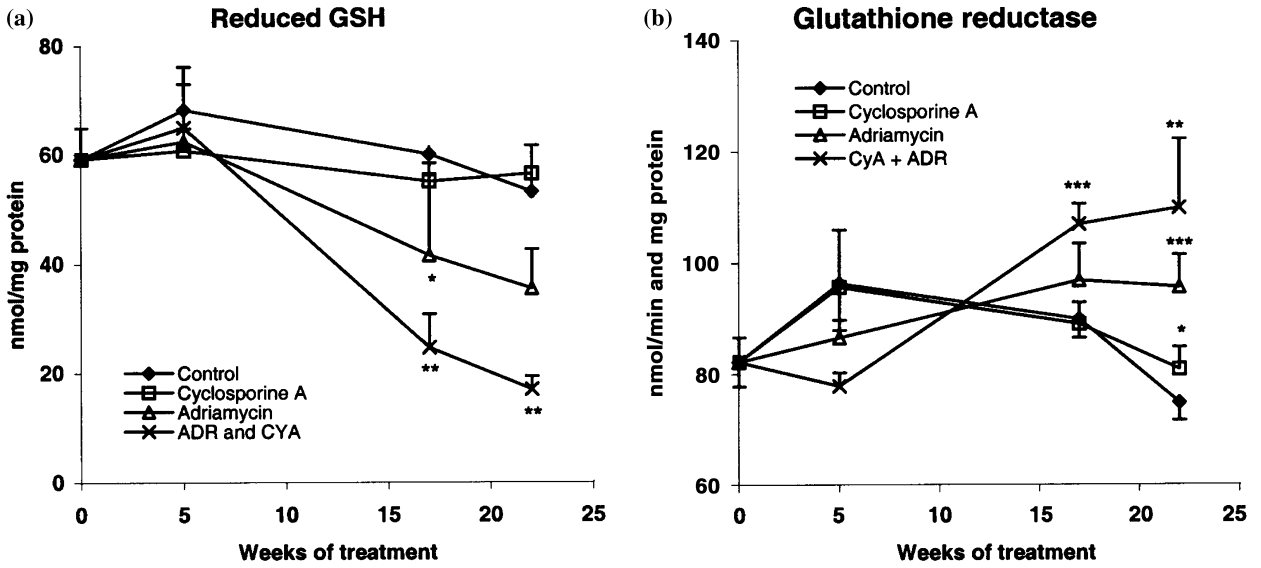


Figure 5 (a) Levels of reduced glutathione (GSH) in rat liver cytosol isolated before and after treatment with cyclosporine A and/or adriamycin for 5, 17 or 22 weeks. Control rats (♦), and rats treated with cyclosporine A (□), adriamycin (△), and a combination of adriamycin and cyclosporine (x). Where indicated, the results are significantly different from those of control rats at the levels of $*P < 0.05$ and $**P < 0.01$, as determined using Student's *t*-test. (b) Activities of glutathione (GSH) reductase in rat liver cytosol isolated before and after treatment with cyclosporine A and/or adriamycin for 5, 17 or 22 weeks. Control rats (♦), and rats treated with cyclosporine A (□), adriamycin (△), and a combination of adriamycin and cyclosporine (x). Where indicated, the results are significantly different from those of control rats at the levels of $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, as determined using Student's *t*-test.

decrease in GSH levels respectively. After 22 weeks the GSH levels in these groups were further decreased compared with the control group (Fig. 5a).

The activity of GSH reductase in the different experiments is shown in Fig. 5b. Only slight changes were observed after 5 and 17 weeks of treatment. However, after 22 weeks of treatment with adriamycin alone or cyclosporine A and adriamycin in combination, the activity of GSH reductase was significantly increased in experimental compared with control animals.

Discussion

One of the major difficulties encountered in treating human HCC by OLT is the early dissemination of tumour cells. It has been suggested that disseminated cells pass through a resting (G0) state, during which time they are unaffected by chemotherapy. If this is indeed the case, factors that will cause cells to leave their resting state and enter into the cell cycle, together with factors that regulate the growth of metastases will be decisive with respect to the length of the recurrence-free survival period and the possibility of a permanent cure.

Growth factors are known to stimulate cells to move from the G0 phase into the G1 phase. In the clinical transplant situation, release of liver regenerative promoting factors can be anticipated, for example, when there is a small-for-size graft–recipient relationship. Other circumstances which might induce a regenerative burst includes the ischaemia–reperfusion injury, rejection episodes or other non-immunological, parenchymal destructing events such as chronic infections or circulatory disturbances. Our data suggest that also adriamycin causes hyperplasia and exerts a stimulatory effect on non-malignant liver cell proliferation, probably through direct cytotoxicity with concomitant growth factor release. If certain tumour cells possess or develop resistance to the cytotoxic effects of adriamycin in a situation where the growth regenerative 'pressure' is high, selection of resistant, growth factor-sensitive tumour cells will occur, causing the tumour(s) to grow more rapidly than in untreated individuals.

This may explain the paradoxical stimulation of tumour growth by adriamycin seen in one of the treatment groups in this study.

An interesting finding in this experiment is the difference in tumour response to adriamycin between the second and the third group of animals. It seems that the tumour morphology is of utmost importance for the outcome. In this study a high grade of nuclear dysplasia and a rapid tumour cell proliferation rate were associated with a better response to adriamycin than was a more slowly growing tumour. If tumour morphology is indeed important, one might hypothesize that there is a primary

difference in the cellular defence against adriamycin between different kinds of tumours. The differentiated tumour cells would then be highly resistant to the toxic compound, a property that is more or less lost during dedifferentiation and development of the poorly differentiated tumour. However, the resistant cells may still be able to respond to growth-stimulating signals which give prerequisites for a selective clonal growth. This hypothesis is supported by experimental data from animal liver tumour models [26,27]. Regarding the experiment with the tumour that was relatively well-differentiated, no conclusions can be drawn, as the tumour establishment was generally poor in all groups including the controls.

The mechanisms for development of drug resistance are probably multifactorial but alterations in the antioxidative defence system may play an important role. In this study we explored the effect on different antioxidants exerted by cyclosporine A and/or adriamycin in the non-tumorous liver. Unfortunately, we do not have data from the different tumour tissues, which might have been given more direct information. However, as cancer development is a continuous process originating from the normal hepatocyte, we choose the liver as a 'surrogate' marker. The cytotoxic effects of adriamycin, which is known to be metabolized by cytochrome P450 systems with the concomitant formation of reactive oxygen species (ROS), led to oxidative stress and cell damage [28]. As cyclosporine A is known to inhibit the efflux of drugs via the pgP170 pathway, combined treatment with adriamycin may result in increased oxidative stress because of higher intracellular levels of the drug. In cells constantly exposed to ROS, antioxidants such as ubiquinol, α-tocopherol and GSH play an important role in preventing cell damage and death [29]. The lipid-soluble antioxidants α-tocopherol and ubiquinol protect cell membranes from lipid peroxidation [30]. The cyclosporine dose chosen in this experiment was rather low in order to avoid severe organ toxicity when combined with adriamycin. In spite of this low dose our results showed significantly increased levels of these antioxidants after 17 or 22 weeks of treatment with the combination of adriamycin and cyclosporine compared with treatment with adrimaycin or cyclosporine alone. We suggest that this induction is caused by long-term exposure to ROS and that it is reasonable to believe that this effect may be even more pronounced with a higher cyclosporine dose, mimicking the clinical setting.

We know from other experimental models that premalignant neoplastic liver nodules are more resistant to the drugs than are the surrounding non-neoplastic hepatocytes [26,27]. As mentioned, we do not yet have data on the inducibility of antioxidants in hepatomas by adriamycin, but we cannot exclude the possibility that cellular defence mechanisms may be induced or superinduced also in neoplastic tissue, a capacity which might then be lost during the pathway of dedifferentiation. It is therefore reasonable to suggest that some, preferable well-differentiated, neoplastic hepatocytes/tumours with elevated defence against lipid peroxidation in combination with increased cytosolic GSH would be more resistant to certain treatments compared with more aggressive dysplastic tumours or even non-neoplastic hepatocytes.

We therefore conclude that simultaneous treatment with cyclosporine A and adriamycin in a situation with remnant HCC cells after OLT may be hazardous because of increased liver toxicity and risk for the creation of a 'chronic' growth stimulatory environment. Due to increased cellular resistance promoted by, for example, cyclosporine A, certain tumour clones may escape from the adriamycin cytotoxicity with paradoxical stimulation of tumour growth as a result. This should be taken into consideration when designing protocols for adjuvant systemic chemotherapy in liver transplantation for HCC.

Acknowledgements

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