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Doxorubicin treatment of rabbit renal VX-2 carcinoma: nephrotoxicity, serum parameters and weight

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Abstract Serum electrolytes, creatinine, urea, protein, albumin, bilirubin and glucose were examined every 4 days until time of death in rabbits with VX-2 carcinoma implanted in one kidney. The rabbits were treated with doxorubicin, nephrectomy or combinations thereof and observed for up to 1 year. Rabbits treated with doxorubicin only showed a slight creatinine rise initially, but over time creatinine reached almost the same concentration as that in nephrectomized rabbits receiving equivalent doses of doxorubicin. Creatinine concentrations increased significantly above the normal range following nephrectomy combined with doxorubicin. Doxorubicin nephrotoxicity in rabbits occurs at lower doses than previously reported. In all rabbits the parameters except creatinine remained stable within the established normal ranges, except for the last 4 days before time of death in the animals with metastatic disease. Weight loss was the best parameter for making a prognosis for an individual rabbit, since peak weight was noted 16–20 days before death. In experimental work with VX-2 carcinoma, weight is thus the most important indicator of the time at which rabbits not responding to treatment can be put to death to avoid unnecessary suffering before the end of the experiment.

Key words Kidney neoplasm · Combined modality therapy · Doxorubicin treatment · Nephrectomy · Creatinine · Electrolytes

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In previous studies of survival after various treatment modes [5], we found that rabbits with progressive disease did not look ill until the terminal stage, shortly before being put to death. In order to predict the outcome somewhat earlier, stimulated by a perusal of the literature that revealed that rabbits with i.m. implanted VX-2 carcinoma develop hypercalcemia [14, 16–18, 20, 22, 23], we decided to investigate whether changes in serum biochemistry and weight could enable progressive disease to be detected in rabbits that did not respond to therapy, especially in those that appeared healthy. In pilot studies we found that the doxorubicin nephrotoxicity in our breed of rabbits seemed to occur after lower doses than previously reported [4, 11]. We thus decided to study the effect of doxorubicin combined with nephrectomy as a potentially very effective model for such a study. In summary, our aim was (1) to find reliable prognostic factors to predict outcome before the terminal stage in rabbits not responding to doxorubicin treatment and (2) using one of the treatment models to determine the doxorubicin threshold dose for nephrotoxicity.

Material and methods

Sixty-six male and female French Burgundy/Chinchilla hybrid rabbits (mean weight 2.9 ± 0.6 kg, age 6–12 months) were used. The VX-2 carcinoma was maintained by serial passages of approximately one million cells into both hind limbs of donor rabbits every 12–14 days. Tumor implantation and treatment were performed with the animal under general anesthesia. In control animals sham injections of 0.015 ml saline were performed in the same manner as for implantation of tumor cells. With the exception of saline-implanted controls, all rabbits were subjected to i.v. cytostatic drug therapy, nephrectomy or a combination of the two. A detailed description of groups, method of tumor implantation, surgical technique and treatment have been published [5].

There were a total of eight groups: seven with implanted renal VX-2 carcinoma and one control group with saline implants (Table 1). Six groups, each containing eight rabbits, were treated

with either doxorubicin (DOX) (Adriamycin, Carlo Erba, Italy) and/or nephrectomy. The total DOX doses used were 2, 4 and 5.5 mg/kg, equivalent to 30, 60 and 85 mg/m^2, respectively. Two control groups, each containing nine rabbits, were left untreated; one had sham surgery and saline injection in the kidney, and the other had implantation of VX-2 carcinoma in the kidney.

After implantation until death the rabbits were monitored daily for signs of illness. Blood samples (approximately 3–5 ml) for biochemical analysis were obtained from a marginal ear vein every 4th day at the same hour (between 9 and 10 a.m.) to avoid possible diurnal variation. The rabbits were not fasted before bleeding. At the same time, weight, hematocrit and leukocyte counts were checked. Blood for biochemical analysis was sampled in glass tubes containing ethylenediaminetetraacetic acid (EDTA) (1 mg/ml blood). The samples were kept for approximately 1 h at room temperature, and then for approximately 1 h in a refrigerator before centrifugation, after which the serum was separated, and the serum samples stored at −70 °C until analysis. Electrolytes (Na^+, Cl^-, K^+, Ca^{2+}, $HPO4^{2-}$), bilirubin, creatinine, glucose and urea were measured using the module chemistry of the Astra 8 autoanalyzer (Beckman Instruments, USA). Sick-looking rabbits and rabbits with progressive weight loss exceeding 10% of their maximum weight were put to death with an overdose of pentobarbital. Cured rabbits were killed following 12 months observation.

The normal range of all parameters was established as mean ± 2 standard deviations (SD) of all participating rabbits on day 0 before any manipulations were made. Reported results are expressed as mean ± 1 SD unless otherwise stated. The paired *t*-test was used to test for the statistical significance of changes within the groups. The unpaired *t*-test was used to test for the statistical significance of changes between the groups.

Table 1 Overview of treatment groups. Treatment result for each group is expressed in parenthesis as the number of cured/number of diseased rabbits in the group

Group	Treatment
0	Control, saline implantation in kidney (9/0)
1	Tumor implantation. No treatment (0/9)
2	Nephrectomy <30 min after tumor implantation (1/7)
3	DOX 2 mg/kg i.v. immediately before tumor implantation + nephrectomy <30 min after implantation (2/6)
4	DOX 2 mg/kg i.v. immediately before tumor implantation + nephrectomy <30 min after implantation + DOX 2 mg/kg i.v. 72 h after tumor implantation (2/6)
5	DOX 2 mg/kg i.v. immediately before tumor implantation + nephrectomy <30 min after implantation + DOX 1.75 mg/kg i.v. × 2, 48 h and 96 h after tumor implantation (2/6)
6	DOX 2 mg/kg i.v. immediately before tumor implantation (4/4)
7	DOX 2 mg/kg i.v. immediately before tumor implantation + DOX 2 mg/kg i.v. 72 h after tumor implantation (4/4)

Results

With the exception of group 5, where six of eight rabbits were put to death because of DOX intoxication, all rabbits that appeared sick or lost more than 10% of maximum recorded weight had extensive metastatic disease at autopsy. All rabbits that were killed after 1 year's observation were healthy, with no signs of malignant disease at autopsy. In Table 2 are shown the serum biochemistry control levels before tumor implantation, at peak weight (4 days before death for cured rabbits) and at the terminal examination. Rabbits that were cured after treatment gained a median of 30% of their weight during the observation time, the same as the controls (Fig. 1). Rabbits that had to be killed due to progressive disease also gained some weight, reaching peak weight 16–20 (median 17) days before death. The body weight started to decrease before any other change could be recorded and was the first sign of illness. Out of 36 rabbits that were put to death because of metastatic disease, seven had muscular invasion or metastases and five had macroscopic

Table 2 Biochemical constituents in serum from Chinchilla/French Burgundy hybrid rabbits, normal control level before tumor implantation, after renal implantation of VX-2 carcinoma during period of maximum weight (mean of three recordings) and with advanced metastatic disease before death

	Control before tumor implantation (n = 66)		Healthy looking rabbits at maximum weight (n = 66)		Metastatic disease before death (n = 36)	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Weight (kg)	2.9 ± 0.6	2.3–4.2	3.2 ± 0.6	2.0–4.2	2.7 ± 0.5	1.7–4.1
Leukocytes (10^3/μl)	10.4 ± 3.8	9.8–24.1	12.2 ± 4.0	6.1–22.0	48.0 ± 40.2	10.4–243.2
Hematocrit (%)	39 ± 2	34–45	38 ± 3	33–43	38 ± 5	27 ± 47
Sodium (mmol/l)	144 ± 3	143–149	143 ± 4	132–153	143 ± 7	114–153
Potassium (mmol/l)	3.6 ± 0.3	3.3–4.2	3.7 ± 0.3	3.0–4.3	4.8 ± 2.1	2.7–13.6
Chloride (mmol/l)	101 ± 3	100–108	102 ± 4	93–112	103 ± 6	84–112
Calcium (mmol/l)	3.44 ± 0.20	3.35–3.78	3.43 ± 0.19	2.98–3.70	3.39 ± 0.31	2.93 ± 4.30
Phosphate (mmol/l)	1.86 ± 0.31	1.71–2.52	1.66 ± 0.31	1.10–2.29	2.06 ± 0.77	1.16–5.26
Creatinine (μmol/l)	82 ± 12	74–118	97 ± 21	58–134	144 ± 49	61–324
Urea (mmol/l)	6.9 ± 1.9	8.6–12.9	6.2 ± 1.3	3.9 ± 8.6	14.1 ± 8.4	3.9–50.0
Protein (g/l)	49 ± 3	48–55	48 ± 4	39–56	47 ± 4	35–57
Albumin (g/l)	39 ± 3	39–43	37 ± 5	21–43	30 ± 4	21–42
Bilirubin (μmol/l)	2.2 ± 0.8	2–4	2.6 ± 1.3	1–8	2.6 ± 1.3	1–8
Glucose (mmol/l)	10.1 ± 2.1	10.5–18.3	8.5 ± 1.2	6.4–11.1	11.6 ± 9.6	4.3–60.8

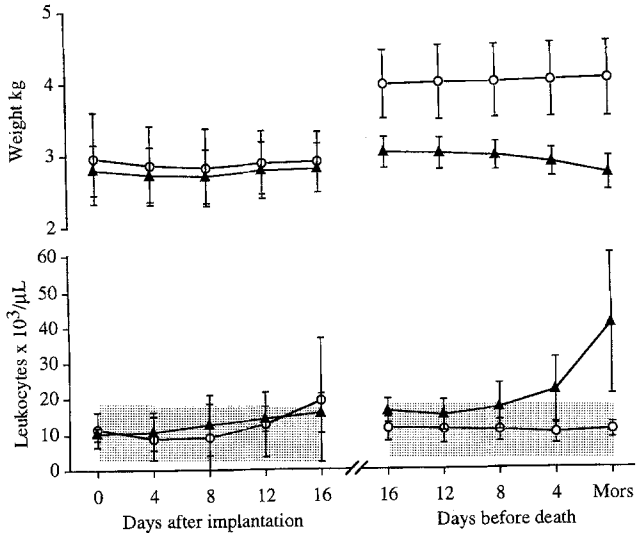

Fig. 1 *Upper*: weight changes in cured (-○-) and diseased (-▲-) rabbits. *Lower*: leukocyte changes in cured and diseased rabbits

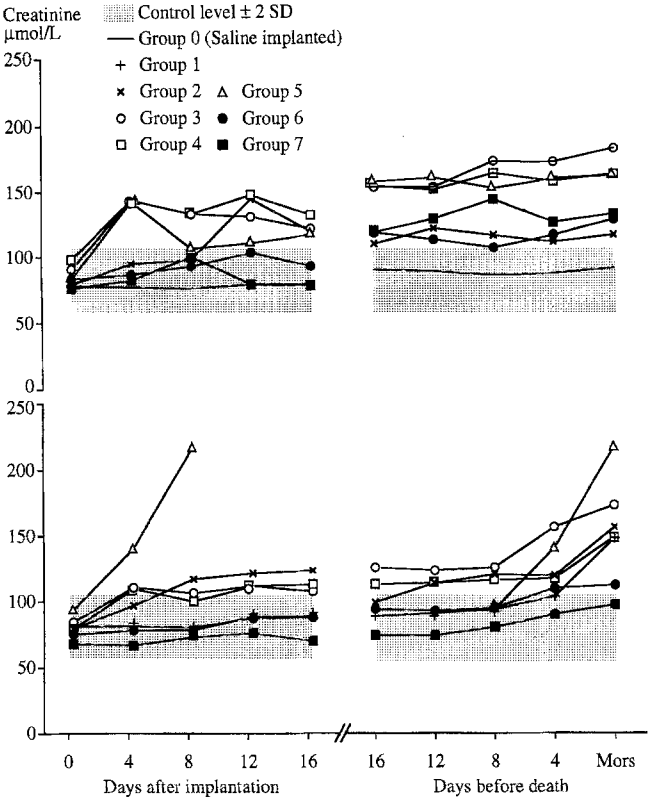

Fig. 2 Creatinine changes in rabbits treated with nephrectomy and/or doxorubicin. *Upper*: saline-implanted and cured rabbits. *Lower*: diseased rabbits

bone metastases detected at autopsy. Only two rabbits had hypercalcemia; both were in the terminal stage with widespread metastases to the lungs and mediastinum. One of them had muscular metastases as well but none had skeletal metastases. The calcium level had been within the normal range 4 days before death. Serum sodium, potassium, chloride, calcium, phosphate, total protein, albumin, urea, bilirubin and glucose remained within the normal range without statistically significant changes until death in all cured and saline-implanted control rabbits. In rabbits that eventually succumbed to metastatic disease, these parameters remained stable within the normal range until 4 days before death.

Creatinine

Creatinine changes are shown in Fig. 2. In both saline-implanted control rabbits (group 0) and cured rabbits that were treated with nephrectomy alone (group 1), creatinine and urea remained within the normal range throughout the observation period. In all nephrectomized rabbits receiving DOX (groups 3, 4 and 5), there was an initial steep rise in creatinine of 38%–54% ($P < 0.001$), which remained pathologically elevated until death in both cured and non-responding rabbits. In rabbits suffering from DOX toxicity (group 5), creatinine levels increased further to 97% above the control level by days 7–9 ($P < 0.02$), at which time six of eight rabbits were put to death. In surviving, cured rabbits from groups 3, 4 and 5 creatinine continued to rise, and was 66%–101% above the control level at death ($P < 0.01$).

Rabbits that were treated with DOX alone initially had only a slight increase in creatinine of 14% and 20% following 2 and 4 mg/kg DOX (groups 6 and 7), respectively. However, creatinine continued to increase gradually and at the time of death was on average 51% (group 6) and 60% (group 7) above the average control level. After equivalent doses of DOX, creatinine elevation was significantly greater in nephrectomized (groups 3 and 6) than in not nephrectomized rabbits (groups 4 and 7). This difference was statistically significant at varying levels throughout the observation period ($P < 0.001$–0.05). There was no significant difference in creatinine elevation regardless of whether 2 or 4 mg/kg DOX had been administered, either in nephrectomized (groups 3 and 4) or not nephrectomized rabbits (groups 6 and 7), or in cured and diseased rabbits. Serum urea remained within the normal range following treatment and did not increase significantly until the rabbits were in the terminal stage.

Mean leukocyte count before tumor implantation was $10.4 \pm 3.8 \times 10^3/\mu l$ (Table 2), consistent with previous results from our laboratory [5], and that reported by others in New Zealand white rabbits [6, 10]. Cured rabbits and saline-implanted controls maintained stable, normal leukocyte counts during the observation time, with the exception of a slight and short-lasting (3–4 weeks) reduction immediately after DOX treatment. In rabbits with advancing metastatic disease, leukocyte counts remained within the normal range until 8 days before death, when the leukocyte counts increased up to 4 times the control level (Fig. 1).

Discussion

Experimental animals appear to be more sensitive to the nephrotoxic effects of DOX than humans [21]. Nephrotoxic effects of DOX with altered renal function and morphology are rare in patients. Myelosuppression, stomatitis and gastrointestinal disturbances are the most common clinical toxic effects and the incidence of DOX-induced cardiotoxicity depends on the total cumulative dose. Myocardial and renal toxicity of DOX have previously been described in rabbits [9, 11, 15, 21]. Dose/response studies of chronic DOX toxicity in rabbits have shown an increased incidence and severity of cardiomyopathy and nephropathy at cumulative doses exceeding 100 mg/m^2 [4, 11]. Using a drug fluorescence method, Bachur et al. [1] demonstrated that in rabbits 17% of i.v. administered DOX was excreted via the bile and only 2% in the urine within 8 h after injection. In the urine unchanged DOX accounted for 62%, doxorubicinol for 35% and polar metabolities for about 2% of the fluorescence.

In a pilot study we found that a total dose of 6 mg/kg or 100 mg/m^2 of DOX administered in 2 mg/kg i.v. fractions on days 9, 11 and 13 after tumor implantation resulted in toxic complications and death in six of eight rabbits 7–9 days after the treatment was concluded. However, a cumulative dose of 5.5 mg/kg or 85 mg/m^2 was well tolerated when administered i.a. or i.v. in three or six fractions from day 9 to day 14. This dose, however, was too high when administered in combination with unilateral nephrectomy. A DOX dose of up to 4 mg/kg, equivalent to 60 mg/m^2, was well tolerated when combined with unilateral nephrectomy.

Mean serum creatinine concentration before tumor implantation and treatment (Table 2) was in concordance with the normal range reported in most studies of New Zealand White rabbits [6, 19, 24], but lower than that reported by Kozma et al. [10]. Following nephrectomy combined with administration of DOX, a sharp elevation of creatinine was noted. There was no correlation between creatinine increase and the amount (number and size) of metastases in the remaining kidney. Higher creatinine levels in groups that were treated with combined DOX administration and nephrectomy when compared with DOX alone may be explained by a greater load, with subsequent increased toxic effect on the remaining kidney. However, in the cured rabbits there was no statistically significant difference in the degree of creatinine elevation regardless of whether 2 or 4 mg/kg DOX had been administered, either when comparing the nephrectomized or the not nephrectomized rabbits. This may be explained by the small sample size or individual variations in response to DOX, but toxic substances from a massive tumor burden may play some additional role in kidney toxicity in those rabbits that succumbed to the malignancy.

Rabbits with i.m. implanted VX-2 carcinoma develop hypercalcemia, and have frequently been used in experimental studies as a model for human paraneoplastic hypercalcemia syndrome. Hypercalcemia in rabbits 2–3 weeks following i.m. VX-2 carcinoma implantation has been associated with increased osteoclastic bone resorption in the absence of bone metastases or bone destruction by the tumor [7, 16, 19, 22].

In most of the studies in which VX-2 carcinoma has caused hypercalcemia, the tumor was implanted i.m. in a hind limb [2, 3, 7, 13, 16–20]. Young et al. [22] investigated VX-2 carcinoma implanted both i.m. and i.p. After i.m. implantation, hypercalcemia developed in 2–3 weeks. Analysis of serum immediately premortem showed 45% of rabbits to be hypercalcemic. However, no hypercalcemia was seen after i.p. tumor implantation of VX-2 carcinoma. They proposed that VX-2 carcinoma cells need a close association with skeletal tissues or muscles in order to induce hypercalcemia and postulated that a hypercalcemic factor released by the tumor cells is inactivated after passage through the systemic circulation. This hypothesis was supported by Rice et al. [12] using a transplantable hypercalcemic Leydig's cell tumor of the Fischer rat. They found that hypercalcemia did not occur when the tumor was implanted in the spleen. Support for this hypothesis may also be found in our study with VX-2 carcinoma implanted in the kidney, where only 2 of 36 rabbits demonstrated hypercalcemia.

In the present study no changes occurred in the serum electrolytes until the terminal stage. From serum creatinine data it appears that DOX-induced nephrotoxicity may occur at lower doses in rabbits than previously reported in the noncured, nephrectomized rabbits. The earliest detectable sign of treatment failure was a cease in weight gain or weight loss, which occurred between 16 and 20 days before death. Rabbits that were cured as a result of the treatment gained on average 30% weight during the observation time. With advancing disease the nonresponding rabbits developed leukocytosis, indicating pulmonary metastases, which were confirmed at autopsy in accordance with the results of Hough Jr. et al. [8].

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