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The Life Shortening Effects of Treatment with Doxorubicin and/or Local Irradiation on a Cohort of Young C3Hf/Sed Mice

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The long-term consequences of treating a cohort of C3Hf/Sed mice in early life with either local-field single dose radiation, systemic doxorubicin, or both, are reported in this study. Significant life shortening was observed in all treatment groups. Median survival times (days) from time of treatment were: control, 690; 35 Gy, 560; 70 Gy, 460; 5 mg/kg doxorubicin, 580; 10 mg/kg doxorubicin, 350; 35 Gy + 5 mg/kg doxorubicin, 510; 70 Gy + 10 mg/kg doxorubicin, 310. Mice receiving hind limb irradiation died principally from induced sarcomas in a dose dependent fashion (80% after 70 Gy and 55% after 35 Gy). Those treated with doxorubicin alone showed an increase in the actuarial incidence of spontaneous malignancies but died mainly from non-malignant causes. Histological examination did not reveal any characteristic cardiac, renal or pulmonary lesions. Doxorubicin did not increase the rate of development of radiation induced sarcomas in mice treated with combined modality.
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INTRODUCTION

THE LONG-TERM sequelae of radiation therapy are well recognised [1, 2]. Localised irradiation may, depending upon dose, site and volume, shorten the life expectancy of patients. This untoward result is due to induction of tumour and to various non-malignant events. The late consequences of chemotherapy have been less well defined in either laboratory animal systems or in man. There is currently a widespread practice in oncology to give adjuvant chemotherapy and aggressive multidrug combinations to patients with locoregional disease only [3]. This paper reports the findings of a prospective study on a cohort of healthy C3Hf/Sed mice treated in early life with either local-field irradiation, systemic doxorubicin or both. Each of these therapeutic modalities reduced the lifespan.

MATERIALS AND METHODS

Animals

The cohort of male and female C3Hf/Sed mice used in these experiments were bred and maintained in our defined flora and pathogen-free colony [4]. Mice were 10–12 weeks of age when entered into the experiment. They were fed sterile Lab Chow and acidified water *ad libitum*.

Irradiation

Local irradiation was given to the right hind limb using a ^{137}caesium irradiator with parallel opposed, circular, 3 cm fields [5]. The dose rate was 7.6 Gy/min.

Doxorubicin

This was reconstituted from a powdered preparation (Adria Pharmaceuticals) and administered intraperitoneally in a volume of 0.5 ml. Two dose levels were chosen: 5 and 10 mg/kg body weight. The $LD_{10/30}$ for C3Hf/Sed mice is 9.8 mg/kg, the $LD_{50/30}$ 12.8, and the $LD_{90/30}$ 16.8. No significant sex difference was observed for these values.

Experimental design

The mice were randomised into 7 groups of 40–60 animals. One group remained untreated and served as controls; the other six were treated with single dose irradiation or doxorubicin, or with combined radiation and doxorubicin as follows: group (1) 35 Gy to the right hind limb, group (2) 70 Gy to the right hind limb, group (3) 5 mg/kg doxorubicin intraperitoneally, group (4) 10 mg/kg doxorubicin, group (5) 5 mg/kg doxorubicin + 35 Gy local irradiation (4 h later) and group (6) 10 mg/kg doxorubicin + 70 Gy local irradiation (4 h later).

Follow-up

Control and treated animals were inspected three times weekly throughout the remainder of their lives. Ailing animals were killed to avoid suffering, and autopsies performed. A record was kept of the macroscopic findings and any obvious lesions underwent biopsy. Biopsy specimens were taken routinely from the hearts, lungs and kidneys of all mice that died. One of the authors (S.M.D.) performed histological examinations on haemotoxylin and eosin preparations of these tissues.

Analysis

As this was a study on the long-term sequelae of therapy, mice dying in the first 30 days post-treatment were excluded from the anaysis. This occurred in 11% of animals receiving the higher dose of doxorubicin and in none of the other groups. Survival and the development of both radiation induced and spontaneous tumours were analysed using standard Kaplan–Meier actuarial methods [6]. Data were plotted at 50 day intervals and mice dying or censored within that time are referred to the end of the period. Survival curves were compared using the Mantel-Haenszel method [7].

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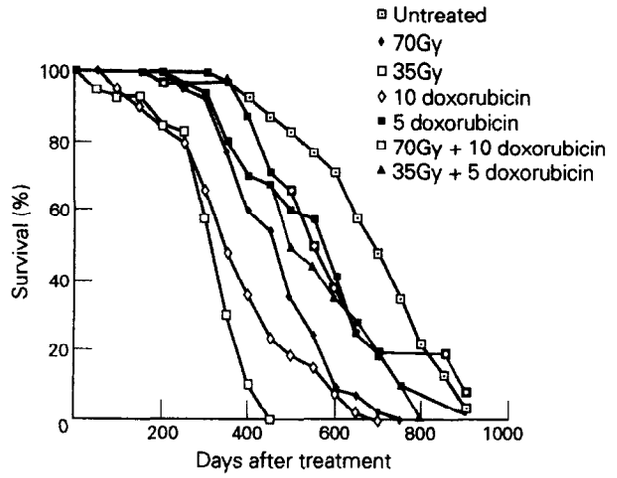
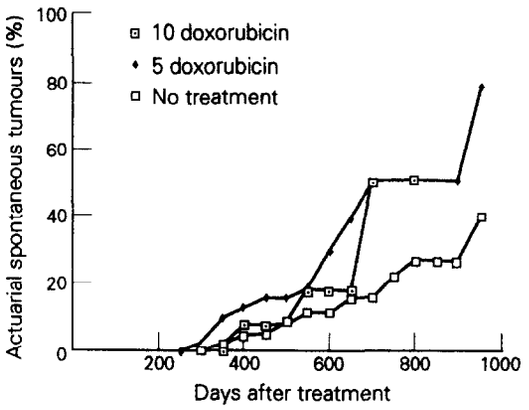


Fig. 1. Survival of C3Hf/Sed mice following treatment with local irradiation and/or intraperitoneal doxorubicin at 10–12 weeks of age. 5 doxorubicin = 5 mg/kg doxorubicin, 10 doxorubicin = 10 mg/kg doxorubicin.



Numbers at risk

No treatment	60	60	60	57	53	46	31	15	8
10 mg/kg doxorubicin	41	37	33	20	10	6	1	0	
5 mg/kg doxorubicin	42	42	40	30	26	18	8	5	4

Fig. 2. Cumulative actuarial incidence of spontaneous tumour development in C3Hf/Sed mice treated with intraperitoneal doxorubicin at 10–12 weeks of age.

RESULTS

The survival of mice in all treatment groups was significantly ($P < 0.05$) shorter than that of controls (Fig. 1 and Table 1). The greatest life shortening effect was seen in mice treated with both high-dose doxorubicin and 70 Gy irradiation. The survival of these mice was shorter than that of mice treated with either high-dose doxorubicin or 70 Gy irradiation alone; this difference did not reach statistical significance. Mice receiving 70 Gy died with median survival times shorter than those receiving 35 Gy. Similarly, mice in the high-dose doxorubicin group died more rapidly than those in the lower dose group.

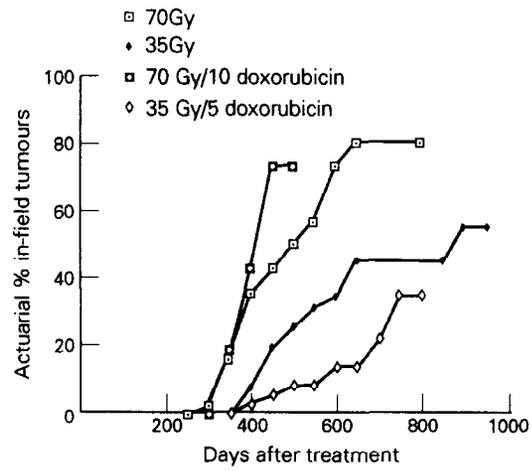
The ultimate cause of death depended upon the initial treatment given (Table 1). Control animals died from either spontaneous malignancies (principally mammary, bronchial and hepatic carcinomas) or from non-malignant causes in a ratio of approximately 1:4.

Doxorubicin increased the proportion of non-malignant deaths in a dose-dependent fashion. In Fig. 2 actuarial analysis shows that, when non-malignant deaths were censored, doxorubicin treatment led to an increase in the incidence of spontaneous tumours. No obvious dose relationship was seen at the two levels tested and the increase was only significant for the 5 mg/kg doxorubicin group ($P < 0.025$). Figure 3 demonstrates that local irradiation hastens death by the induction of in-field tumours (9 of 9 histologically examined were high grade fibrosarcomas). While a clear dose-response relationship was seen in the development of radiation induced tumours there was no enhancement by the doxorubicin. The combination of 35 Gy and 5 mg/kg doxorubicin led to a significantly lower cumulative

Table 1. Median survival and causes of death among C3Hf/Sed mice treated at 10–12 weeks of age with systemic doxorubicin and/or local hind limb irradiation

Group	No.	Median survival (days)	Deaths: In-field tumours	Deaths: Spont. tumours	Deaths: NMD
Untreated	60	690	–	13	47
35 Gy	37	560	16	6	15
70 Gy	44	460	27	6	11
5 mg/kg	42	580	–	16	26
10 mg/kg	41	350	–	4	37
35 Gy + 5 mg/kg	42	510	9	5	28
70 Gy + 10 mg/kg	40	310	18	2	20

Spont. = spontaneous, NMD = non-malignant disease.



Numbers at risk

70 Gy	45	45	42	27	16	4	1	0	
35 Gy	37	36	36	33	25	14	7	7	3
70 Gy/10 doxorubicin	38	37	33	12	0				
35 Gy/5 doxorubicin	41	41	41	36	21	15	7	0	

Fig. 3. Cumulative actuarial incidence of in-field tumours following irradiation of the right hind limb of C3Hf/Sed mice at 10–12 weeks of age. 5 doxorubicin = 5 mg/kg doxorubicin intraperitoneally 4 h prior to irradiation, 10 doxorubicin = 10 mg/kg 4 h intraperitoneally prior to irradiation.

Table 2. Histological findings in mice dying over the time period 300–600 days post-treatment with 10 mg/kg systemic doxorubicin

Treatment	Heart	Lungs	Kidneys
Control ($n = 8$)	8 normal	8 normal	5 normal 2 focal interstitial calcification 1 focal interstitial pigmentation
Doxorubicin ($n = 11$)	9 normal 1 focal necrosis 1 myxoid degeneration	6 normal 3 atelectasis 1 pneumonia 1 myxoid degeneration	9 normal 1 tubular necrosis 1 focal interstitial calcification

incidence of spontaneous tumours than 35 Gy alone. When doxorubicin and local irradiation were combined, the proportion of spontaneous tumours developing was very low as most animals died from a combination of in-field tumours and non-malignant causes.

The majority of animals receiving high-dose doxorubicin displayed marked cachexia prior to death. A pathological study was performed on organs from a random selection of these and untreated mice dying in middle life (300–600 days) (Table 2). No major histological differences between the hearts, lungs and kidneys of mice in the two groups were detected. Most of the pathological observations listed are non-fatal degenerative conditions. A search was made for the characteristic signs of doxorubicin cardiomyopathy and interstitial nephritis. The former was seen in only 1 mouse of 11 examined, and the latter in none. Blood smears were taken from the 7 mice in the high-dose doxorubicin group alive at > 500 days: dysplasia and neoplasia were not seen. The non-malignant cause of death in the doxorubicin treated groups thus remains unclear.

DISCUSSION

Patients are now living for extended periods following successful treatment of malignancy in early life. This report describes the consequences for a large cohort of mice treated in their youth with doxorubicin, irradiation or both, and followed throughout their natural lifespan.

Doxorubicin is a DNA intercalating agent with activity in a wide range of malignant diseases. In humans the most commonly described toxicities are early, dose-related myelosuppression and focal myocardial degeneration [8]. In rodent models a similar pattern of toxicity is seen with myelosuppression in the first 30 days and cardiac and renal damage up to 200 days after treatment. Neurological and pulmonary toxicities are also described although these are not usually fatal [9, 10]. In our study doxorubicin led to dose-dependent life shortening that resulted, principally, from accelerated non-neoplastic mortality. These deaths occurred 300–600 days after treatment. This is beyond the previously described latency for cardiac and renal toxicity in mice and suggests a different pathogenic mechanism. Disturbed cardiac function, arrhythmias and sudden death have recently been described in a cohort of patients treated with anthracyclines in childhood and followed for 4–20 years [11]. These observations are in keeping with the long-term sequelae that we report in mice and may also explain our inability to demonstrate any specific histological lesion.

Many clinicians are concerned that the carcinogenic nature of some cytotoxic agents may reduce the lifespan of long-term survivors of therapy. Solcia reported an increase in the rate of development of spontaneous tumours in Sprague–Dawley rats following a single injection of doxorubicin [12]. We have shown a similar increase in mice. Neither study demonstrated a dose-response in the range up to 10 mg/kg doxorubicin.

The induction of malignancy by irradiation is well described both experimentally [13, 14] and clinically [15, 16]. In humans the highest incidence occurs in those irradiated in childhood. Doxorubicin potentiates many of the acute effects of radiation on normal tissues [17, 18] but its influence on late effects is less clear [19, 20]. Our results did not indicate that doxorubicin enhanced the carcinogenic action of irradiation. The radiation given in this study was administered in high single doses and is thus not strictly comparable to fractionated radiation given in the clinic. It must, however, be noted that tumour induction differs from other radiation late effects in that there is no evidence that its incidence is reduced by fractionation. The doses of doxorubicin chosen represent levels at which there is significant acute mortality ($LD_{10/30}$ is 9.8 mg/kg) and are biologically comparable to doses given clinically. Patients can receive doses that, in a relative sense, are even higher as antibiotics and haematological support offer successful salvage from the acute toxicity. The treatment may then be repeated after an appropriate interval. Single dose chemotherapy has been given in adjuvant protocols to breast cancer patients [21]. Very high single doses of cytotoxic drugs are given prior to bone marrow transplantation in a range of malignant disorders and it is anticipated that there will be long-term survivors.

In conclusion, the life shortening seen in C3H mice exposed to local irradiation was principally the consequence of induced malignancy. The synchronous administration of doxorubicin did not increase the rate or latency of these tumours. For mice treated with doxorubicin alone the pattern was less easy to assess. An increase in the number of spontaneous malignancies was observed but the majority of deaths were from non-malignant causes.

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Feature Articles

Novel Growth Regulatory Factors and Tumour Angiogenesis

Roy Bicknell and Adrian L. Harris

INTRODUCTION

RESEARCH OVER the past 20 years in many laboratories has established that angiogenesis is an essential component of tumour growth. A variety of experimental systems have shown that tumours do not grow beyond a size of 2–3 mm^3 unless they are able to attract the growth of new capillaries from the existing vascular network. The evidence that growth of solid tumours is angiogenesis dependent has been reviewed by Folkman [1]. In addition, several clinical studies have shown that neovascularisation is a poor prognostic factor in breast [2], cervical [3] and bladder [4] cancer. Apart from their necessity for growth of the tumour, the new blood vessels provide an essential entry route to the vasculature for metastasis of tumour cells. The last 2 years has seen a surge in the number of factors known to stimulate or inhibit angiogenesis. It has become clear that many well characterised growth factors for epithelium are not active on endothelium. Angiogenesis involves proliferation of capillary endothelium. In the healthy adult, endothelial cells are normally held in a quiescent state (an exception occurs during the menstrual cycle), and proliferate only in response to unusual circumstances for example wound healing and in disease states such as tumour vascularisation. It follows that the proliferating capillary endothelial cell offers a unique target for antiangiogenesis therapy [5]. In addition the endothelium may provide a drug resistance barrier that protects the tumour cells from anticancer drugs. Several resistance mechanisms found in tumours are present in endothelial cells, one example is expression of the multidrug resistance gene [6].

This paper reviews results of the last 2 years that are of relevance to tumour angiogenesis and assesses the possibility of antiangiogenic therapy.

ANGIOGENIC FACTORS

Angiogenesis is a complex, multistep process that involves not only endothelial cell proliferation but also digestion of the extracellular matrix surrounding intact capillaries by collagenases and related proteases, endothelial cell migration and differentiation into functioning capillaries.

Several quite different assays of angiogenesis have been used. In addition the component steps of angiogenesis, e.g. cell proliferation, migration or tube formation are often studied separately *in vitro*. Few factors have been examined for activity in each assay, leaving a somewhat complex picture of the precise role of different factors in tumour angiogenesis. Table 1 lists the known polypeptide angiogenic factors and endothelial growth factors, a list which is rapidly increasing. The most studied factor in the context of angiogenesis is fibroblast growth factor (FGF), now known to be a member of a family of at least seven sequence related growth factors (basic FGF, acidic FGF, hst/KS3, int-2, FGF5, FGF6 and keratinocyte growth factor or FGF7) [7], all of which are potentially angiogenically active. Other angiogenic polypeptides which are mitogens for endo-

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