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Original Paper

Perivascular Cell Protection *In Vivo* and Increased Cell Survival *In Vitro* by the Antihypertensive Agent Carvedilol Following Radiation

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Carvedilol, an antihypertensive drug with activity on adrenoceptors as well as on calcium channel activity, has recently been introduced. In the present study we investigated whether carvedilol interacts with the cytotoxicity induced by irradiation *in vitro* as well as *in vivo*. A daily injection of carvedilol in clinically relevant concentrations (3 mg/kg subcutaneously), 4 days before and 3 days after a single radiation dose of 20 Gy significantly decreased the inflammatory reaction in the rat lung, evaluated as number of inflammatory cells in the perivascular area. The density of mast cells was also slightly reduced. *In vitro* studies revealed that carvedilol caused different radio-protective effects, dependent on dose (1–7 Gy) used and cell line studied. The effects were especially pronounced in a malignant mesothelioma cell line (P-31), and somewhat less evident in a prostatic carcinoma cell line (PC-3). No significant effect was seen in a highly radiosensitive small cell lung cancer cell line (U-1690). Thus, carvedilol may under some circumstances interact with radiation-induced tissue reactions, most probably by a direct interaction at the cellular level. The specific explanation to the differences in sensitivity to carvedilol remains to be evaluated, but the known antioxidative properties and/or scavenging of free radicals of carvedilol may be a plausible mechanism of action. Secondary induced alterations in inflammatory response may also be considered. It is suggested that a potential interaction between drugs such as carvedilol and irradiation should be considered for clinical practice.

Key words: irradiation, vasoactive drugs, pneumonitis, rat, cytotoxicity, carvedilol
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INTRODUCTION

SEVERAL VASOACTIVE drugs have been suggested to interact directly or indirectly with cancer treatment and special interest has been devoted to the calcium channel blockers [1–3]. This is mainly due to the reversing effect of calcium channel blockers on p-glycoprotein (p-170)-mediated chemoresistance [4–6]. However, toxic effects have hampered the possibility of achieving therapeutic concentrations in patients [7, 8]. Other drugs with quite different mechanisms of action have also been shown to be of potential interest in modifying the effects of radiation and cytostatics, such as vasodilators and diuretics [9–13].

Carvedilol, an antihypertensive drug with multiple-actions, has recently been introduced. It has β-adrenoceptor antagonist activity, mediates vasodilatation primarily through α_1-adrenoceptor blockade and affects the calcium channels [14, 15]. The antioxidative properties of carvedilol [16–19] might be relevant. Tissue damage following irradiation and some chemotherapeutics involve the generation of free radicals [20]. Thus, there is a potential possibility that the antioxidative capacity of carvedilol can interact with the effects of irradiation on normal tissues as well as with the antitumoral effects.

Therefore, we found it of interest to evaluate whether carvedilol could interact with the toxicity induced by radiation on tumour cells *in vitro* and *in vivo*.

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MATERIAL AND METHODS

Radiation induced pneumonitis

Sprague–Dawley rats, with a mean weight of 390 g at the start of the experiment, were used. The animals were kept under standard conditions at the central animal laboratory. They were divided into one control group and one group treated with carvedilol. Carvedilol was given by daily injections of 3 mg/kg subcutaneously (s.c.), 4 days before irradiation and continued 3 days after the radiation treatment. The control group received the same dosing regimen with vehicle only. Carvedilol (provided by SmithKline Beecham Pharmaceuticals) was dissolved in 100% dimethyl sulphoxide (DMSO), then diluted in 0.9% saline to achieve a final concentration of 3 mg/ml with 55% DMSO in saline. Vehicle-treated animals received the same volume of 55% DMSO in saline. At the time of irradiation, both control and experimental rats were anaesthetised by an intraperitoneal (i.p.) injection (3 ml/kg) of a mixture (v/v) of one quarter midozolam (Dormicum®; Roche, Basel, Switzerland), one quarter fentanyl/fluanison (Hypnorm®; Janssen Pharmaceutical, Beerse, Belgium), and half sterile water. The animals were thereafter placed in a body-shaped container and given a single radiation dose of 20 Gy to the right lung. The dose was chosen from our earlier experiments, demonstrating that 20 Gy caused a pneumonitis within 6–7 weeks [21, 22]. The left lung was protected with a shield of lead. The irradiation was given with photons, 4 MV, dose rate 2.7 Gy/min, focus to skin distance: 67.5 cm. Seven weeks after irradiation, the rats were killed and the lungs were taken out for histological examination.

Morphological analysis

The methods used have been previously described [21, 22]. Lungs and trachea were dissected free and a small plastic tube with a stiff leader inside was inserted into the trachea. The lungs were expanded by instillation of a solution of either Bouins or Histocon, which is used for preparation of samples for cryosectioning. The specimens were stored frozen at -70°C until use. The lungs fixed in the Bouins solution were, after routine preparation, embedded in paraffin and sliced in the sagittal plane. 5 μm thick sections were used for the examination.

Sections were stained with Masson's trichrome, Luna and toluidine blue. Slides stained with Luna and toluidine blue were used to determine the total numbers of cells and total numbers of mast cells in the alveolar interstitium. Four areas of lung tissue were defined as described earlier [21, 22]: lung interstitium (e.g. alveolar septal tissue), alveolar tissue, peribronchial tissue and perivascular tissue. In order to count mast cells, the slides were stained with acid toluidine blue and counter-stained with Mayer's acid haematoxylin. With this fixation and staining technique, the morphology was less clear and it was difficult to differentiate between peribronchial and perivascular tissue. Therefore, with regard to mast cell counting no differentiation between peribronchial and perivascular tissue was made. Ten fields with an accumulated area of 0.113 mm^2 were counted at a magnification of ×480. The results are presented as numbers of cells per mm^3.

Cell culture

Three different cell lines were used, a prostatic carcinoma (PC-3), a malignant lung mesothelioma (P-31) and a small cell lung cancer (U-1690). The prostatic cell line (PC-3) is a poorly differentiated adenocarcinoma that does not respond to androgens, glucocorticoids, epidermal or fibroblast growth factors [23]. It is also highly chemoresistant and expresses Pgp, indicative of multidrug resistance (MDR) [24]. The mesothelioma cell line (P-31) is an aneuploid cell line established from pleural effusions [25], and the third cell line (U-1690) is derived from small cell carcinoma of the lung and is highly radiosensitive [26]. The cells were cultured as a monolayer on plastic Petri dishes, using Eagle's MEM, supplemented with 15% fetal calf serum and antibiotics. The cells were incubated at 37°C in humidified atmosphere of 5% CO_2–95% air.

Irradiation and carvedilol treatment of the cells

Cells were irradiated with 195 kVp X-rays in a field size of 15×15 cm and a distance of 50 cm between radiation source and the bottom of the Petri dishes. The X-ray beam was filtered with a 0.5 mm copper + 0.5 mm aluminium filter. An absorbed dose level between 1 and 7 Gy was delivered during 1.17–8.0 min. Exponentially growing cells were seeded onto new plastic Petri dishes containing nutrient medium and incubated for 4 h before irradiation. The number of cells plated was adjusted according to the dose in such a way that 50–100 clones could be expected to survive. Carvedilol was added 60 min before irradiation. 30 min after irradiation, the medium with the vasoactive drug was removed, medium without test drug was added and the dishes were then incubated for 14 days. Three experiments in triplicate were done. The cells were divided into six groups—group 1: without carvedilol and not irradiated, group 2: without carvedilol and irradiated, group 3: with carvedilol 1.0 μmol/l and irradiated, group 4: with carvedilol 10 μmol/l and irradiated, group 5: with carvedilol 1.0 μmol/l and not irradiated, and group 6: with carvedilol 10 μmol/l and not irradiated. All other conditions were the same for all groups. After incubation for 14 days, the surviving clones were fixed and stained *in situ*. Surviving clones were defined as the percentage of treated clones (treated with carvedilol and/or irradiation) that grew into macroscopic colonies as compared with group 1 (i.e. without drug and not irradiated).

Statistics

Statistical significance of the difference between control and test groups *in vitro* were tested with the Mann–Whitney U-test and Wilkoxon's rank sum test for unpaired samples *in vivo*.

RESULTS

In vivo—*rat lung*

Seven weeks after irradiation, the administration of carvedilol resulted in lower numbers of inflammatory cells, both in irradiated and unirradiated rats (Figure 1 and Table 1). For two groups the difference reached statistical significance: carvedilol particularly decreased the number of inflammatory cells in the perivascular area following irradiation. There was a marked increase in the number of inflammatory cells and mast cells in the irradiated lung compared with the opposite unirradiated lung. The effect was most dramatic for the mast cells because these were absent in the unirradiated lungs.

In vitro—*tumour cells*

The results demonstrated a variation in the effects dependent on the cell line studied. Carvedilol protected in a dose-dependent manner both the lung mesothelioma cell line (P-31)

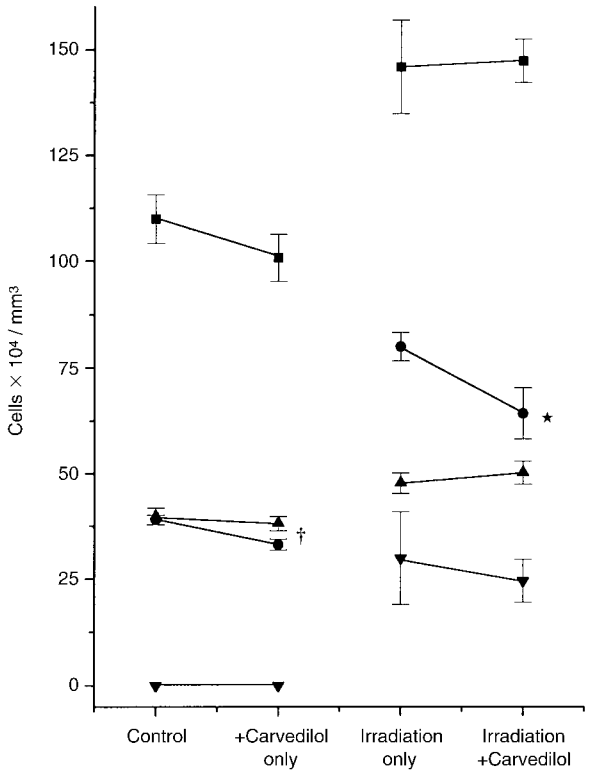

Figure 1. Total numbers of cells and total numbers of interstitial mast cells in rat lung. Controls ($n = 7$), Controls treated with carvedilol (3 mg/kg s.c. ($n = 9$)), Irradiated (20 Gy, single dose ($n = 7$)) and irradiated+treated with carvedilol ($n = 8$). The results are shown as mean ± SEM. Statistical comparisons between the groups were made with the Wilcoxon's rank sum test for unpaired samples. *$P<0.01$ compared to untreated controls. †$P<0.05$ compared to irradiated rats without carvedilol. ■, interstitium; ●, perivascular area; ▲, peribronchial area; ▼, mast cells.

(Figure 2a) and the prostatic cell line (Figure 2b) against irradiation. No direct cytotoxicity was found in unirradiated cells treated with carvedilol compared with untreated cells in these two cell lines. The curves in Figure 2 were fitted according to the linear quadratic model and the values of α and β for all 6 curves are shown in Table 2. The main change resulted from the addition of carvedilol, especially for the malignant mesothelioma cell line. The protection factors obtained from comparing the X-ray dose with and without the drug to achieve equal levels of cell kill ranged from 1.05 to 5.00 for the lung mesothelioma and from 1.09 to 1.36 for the prostatic cancer cell line.

In the highly radiosensitive small cell lung cancer cell line (U-1690) no radio-protective effects were induced by carvedilol (data not shown). The mean surviving fraction after only irradiation (1 Gy) was 49% of controls, and following pre-treatment with carvedilol (1 or 10 μmol/l) 47 and 45%, respectively.

In this cell line carvedilol only, at both 1 and 10 μmol/l, seem to display a direct cytotoxic effect (the survival fractions were 59 and 74% compared with untreated control). Thus, no significant protective effects against irradiation were seen with carvedilol.

DISCUSSION

The present study demonstrated a radioprotective effect of the clinically used antihypertensive drug, carvedilol, in therapeutically used concentrations. The effects were seen both *in vitro* and in irradiated rat lung. *In vivo* the number of inflammatory cells was reduced in the perivascular area, 7 weeks after a single dose of radiation to the rat lung. Moreover, the amount of mast cells was also lower in the carvedilol treated lung tissue, but did not reach statistical significance. Two of three different cell lines were protected *in vitro* dose dependently by carvedilol, a greater effect for 10 μmol/l than for 1 μmol/l.

Table 1. *Total number of inflammatory cells in the rat lung, ± carvedilol (3 mg/kg s.c.) and exposed to a single radiation dose of* 20 *Gy, compared with non-irradiated lung*

	No.	Perivascular area	Peribronchial area	Alveolar area	Interstitium	Mast cells
Irradiated	7	80.0±3.3	47.7±2.4	63.4±9.4	145.9±11.0	29.9±11.0
Irradiated ± carvedilol	8	64.3±6.0★	50.2±2.7	64.2±9.0	147.4±5.1	24.5±5.1
Untreated control	8	39.0±1.1	39.8±2.0	2.8±0.4	109.9±5.8	0.0±0.0
Carvedilol	9	33.1±1.3†	38.1±1.7	2.1±0.5	100.8±5.5	0.0±0.0

Total cells×10^4/mm^3. Mean ± standard error of the mean (SEM). *$P<0.05$, compared with irradiated rats without carvedilol. †$P<0.01$, compared with untreated control rats.

Table 2. *Values of α, β and the range of protection factors after treatment with carvedilol at two concentrations before irradiation (1–7 Gy)*

	α	β	SF 2 Gy	Protection range from isoeffective doses	α-ratios	β–ratios
Prostatic cancer cell line PC-3						
Irradiated	0.146	0.079	0.59			
Irradiated+carvedilol 1 μmol/l	0.130	0.068	0.62	1.09–1.14	1.12	1.16
Irradiated+carvedilol 10 μmol/l	0.077	0.069	0.62	1.14–1.36	1.90	1.15
Malignant mesothelioma cell line P-31						
Irradiated	0.105	0.025	0.73			
Irradiated+carvedilol 1 μmol/l	0.047	0.031	0.84	1.05–2.00	2.23	0.81
Irradiated+carvedilol 10 μmol/l	−0.007	0.031	0.92	1.19–5.00	Very large	0.81

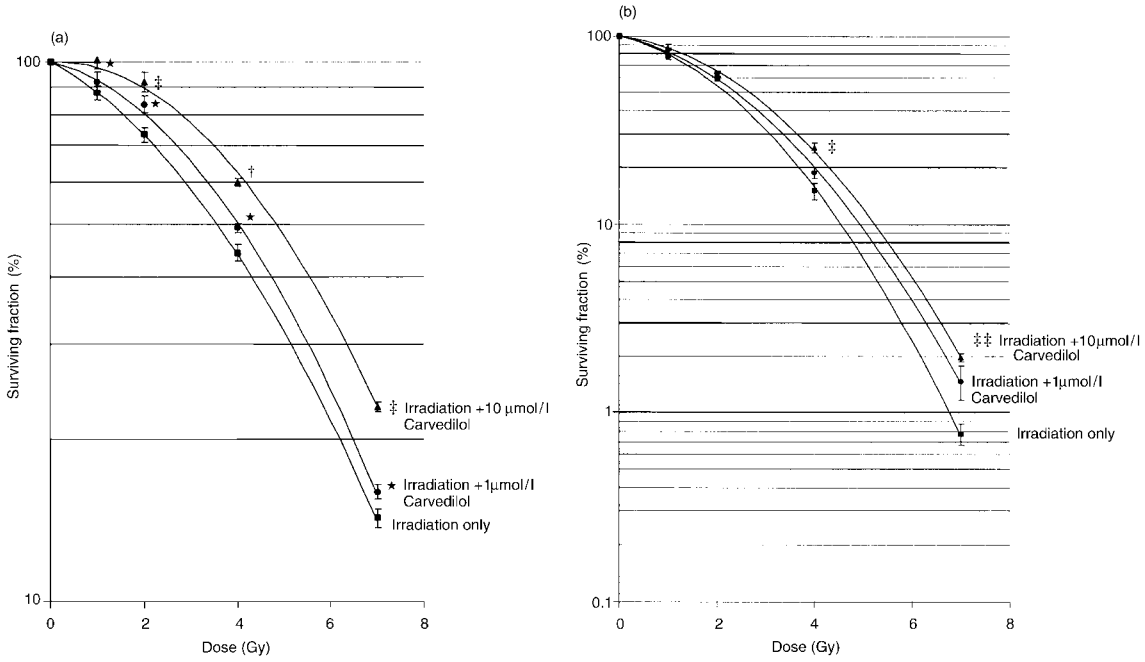

Figure 2. (a) Malignant mesothelioma cell line P-31 and (b) prostatic carcinoma cell line PC-3 survival after pre-treatment with or without carvedilol 1 and 10 μmol/l, 60 min before irradiation with 1–7 Gy compared with untreated controls (100% survival). Results are expressed as mean ± SEM. Curves fitted with the linear quadratic model. The number of experiments was three in triplicate for each radiation dose. *$P<0.05$, †$P<0.01$, ‡$P<0.001$ compared to control.

From the *in vitro* analysis it was evident that the major effect seemed to be on the linear component of the survival curve, producing a greater degree of protection at the lower clinically relevant doses than at doses above 2 Gy. The range of values in Table 2 was derived at different levels of survival by comparing the radiation doses to produce equal damage with or without the drug. The use of the clonogenic assay and graded doses permitted the quantitation of the protection factor. Although there was a wide range for the protection factor derived from each curve because of the different modification of the linear and quadratic components, there appeared to be a greater effect in the mesothelioma than in the prostate cancer cell line. The mesothelioma cell line is more resistant, showing a 20-fold higher survival after 7 Gy in the absence of the drug (compare Figure 2, SF = 0.8 versus 13%). This cell line is known to have high levels of superoxide dismutases, but earlier reports have failed to show any correlation between levels of this enzyme and radiation response [25]. The third cell line U-1690 is very radiosensitive with only a 50% survival after irradiation of 1 Gy. In this cell line, carvedilol displayed a direct toxic effect on unirradiated cells, which neither of the other cell lines showed. The differences in sensitivity to carvedilol are difficult to explain, but add new information about the complexity in evaluating interactions in experimental models. It is obvious that one must carefully interpret such data, and emphasise that variation in toxicity induced by various agents can be completely different depending on cell lines studies.

It is not possible to specify the precise mode of action from the data, but it could result from free radical scavenging at the site of radiation injury, presumed to be the DNA. Two reports have described a neuroprotective effect and myocardial protection due to the antioxidant action and/or the effect of scavenging from free radicals [16, 17]. Carvedilol is known to have a number of different modes of action on cell migration, cell proliferation as well as its antioxidant properties and its calcium channel blocking activity [18, 27, 28]. Vasodilatation induced by carvedilol involves both α and β adrenergic responses. Thus, it may act to suppress repair mechanisms caused by irradiation and other treatment modalities associated with induction of free radicals and damage to membranes and the genome. It is known from other studies with calcium channel blockers that verapamil and diltiazem display radioprotective effects [1, 10]. The suggested effects are believed to be due to a direct inactivation or an interference with the damaging cellular influx of calcium after membrane injury following radiation-induced free radicals. The influence on cytotoxic drug sensitivity could be attributable to calcium pumps, but that is unlikely to be involved in the protection against ionised radiation. No reports have, as far as we know, been published on the interaction of carvedilol and cancer therapy.

An effect of carvedilol was also observed *in vivo*, with the lung being the test tissue. The effect was demonstrable in histological sections 7 weeks after 20 Gy. This is an early time point compared with the time at which functional damage would normally be expressed after bilateral lung irradiation, normally 12–16 weeks. The response is in the acute pneumonitis phase and is associated with an increased number of inflammatory cells (Table 1 and Figure 1). The most dramatic change was seen in the mast cells which were absent in unirradiated lung, but were greatly increased after irradiation both with and without carvedilol. Mast cells have been shown to correlate strongly with the degree of radiation-induced tissue reactions in the lung [21, 22, 29, 30] and salivary glands [9]. A considerable increase in inflammatory cells was also seen in the interstitial space (a 30% increase), and was not significantly different with the addition of the drug. A much smaller effect was seen in the periobronchial space, representing a 15–20% increase after radiation, with or without

drug. The most dramatic effect of the drug was seen in the perivascular space where a significant reduction in inflammatory cells was seen, both in the irradiated and unirradiated lungs. Thus, a reduced inflammatory response was demonstrated in the rat lung using a clinically relevant dose of carvedilol as administered for a week, with irradiation 4 days after the treatment started. The half-life of the drug is known to be 6–7 h, so that daily injections would maintain a reasonable level in the plasma and in the tissues [14]. The irradiation was given 3 h after the injection of the drug, so that a high level would be present at the time of irradiation.

Because a fixed single dose of radiation was used in both arms of the *in vivo* study, it was not possible to quantify the magnitude of the protective effect, but it appeared to differ for different sites within a single tissue. Considering our preliminary data with the superoxide radical generator, pyrogallol, which did not display any of the protective effects of carvedilol, and the fact that in the highly sensitive cell line carvedilol was without any radioprotective effects, the results seen *in vivo*, at least partially, might be explained by a specific anti-inflammatory effect rather than direct radioprotection. In the present observations on lung, it is important to note that the perivascular effect occurred both in the irradiated and in the contralateral unirradiated lung. Since one of the primary target cells for radiation-induced lung injury is probably type 2 pneumocytes, the secondary inflammatory reactions probably represent an altered amount of primary cell damage. The timescale was too early for the response of endothelial cells and for secondary fibrotic reactions and further studies with longer timescales of observation would be needed to determine the functional consequences of these observations made at 7 weeks.

In conclusion, novel data have been presented on the radioprotective action of a new antihypertensive drug, both on tumour cells *in vitro* and perhaps on a normal tissue *in vivo*. The effect differed in magnitude for the three cell lines studied and for different sites within the lung. Since the drug has many different effects [31], it is not yet possible to elucidate the mechanism of this radioprotection, nor the importance of secondary anti-inflammatory responses. It is important, however, to note that this effect occurred at doses that could be administered to cancer patients for concomitant cardiovascular disease. A protective effect on normal tissues could, of course, be beneficial but our *in vitro* studies show that the drug could also provide an undesirable protection for tumour cells, especially at low irradiation dose levels that are used as daily fractions in conventional radiotherapy. Anyhow, further experiments are needed to clarify the effects of this drug in combination with cancer therapy.

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