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THE EFFECT OF RADIATION AND CYTOTOXIC PLATINUM COMPOUNDS ON THE GROWTH OF NORMAL AND TUMOUR BLADDER EXPLANT CULTURES

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Abstract

Using an explant tissue culture model developed by this group for use with human surgical and biopsy specimens, data are presented showing the response of normal and tumor bladder urothelium to radiation in combination with cis- and carboplatin. Cellular response is measured after two weeks in culture as a reduction in the extent of outgrowth from the explant. The outgrowth has been shown to be growing and to be epithelial. Results showed that when either drug or radiation is used singly, the tumour is resistant to treatment while the normal cells are severely affected. However, appropriate combinations of either drug with radiation reverse the unfavourable therapeutic ratio and result in higher tumour cell kill. The model may be useful for investigating mechanisms of radiation/chemotherapy action at the cellular level and, if integrated into appropriate clinical trials, may serve as an easy-to-use in vitro test for optimising single agent or combination therapy regimens.

Key words: Urinary bladder, cell culture, in vitro drug testing, platinum complexes, irradiation.

Normal tissue damage is a considerable problem which limits the dose of tumoricidal therapy which can be given. Methods of coping with the problem usually involve either the use of combinations of drugs with different actions or giving sufficient time for normal tissues to repair or recover. Obviously, neither method is ideal due to the very variable responses of both normal and tumour tissues to any treatment and because of the lack of understanding of all the complex factors involved in the final expression of cellular damage. In view of this it is very surprising that there are so few studies done on the relative response of tumour and normal cells to treatment or on the basic exploitable differences between normal and tumour cell response to a given cytotoxic drug. This problem was referred to by Seymour et al. (1), who where unable to find any in vitro studies using human cells which addressed the problem of relative damage to tumour and normal cells.

There is clearly a need for a rapid, easy assay of tissue response which simulates the in vivo situation as closely as is possible given ethical constraints and technical problems. A system which gives data on response of normal and tumour tissue from the same patient would be most useful.

Our group are developing and attempting to validate an explant model (1–3) which allows pieces of normal and tumour tissue to be treated in culture 24–48 h after explantation and before growth is evident. The cellular growth response can then be measured in vitro over an appropriate time interval ranging from 1 to 4 weeks. Using the model the authors were able to study the response of normal and malignant oesophageal cells to bleomycin in combination with radiation. In the present paper the same technique is used to study the response of bladder urothelium to radiation in combination with the platinum compounds cis- and carboplatin.

Material and Methods

The methods have been described in detail elsewhere (2). Briefly, bladder tissue was obtained from total cystectomy operations.

Normal tissue was obtained from the parts of the resection specimen remote from the tumour and were confirmed as histologically normal by the hospital pathology department. Tissue samples were collected in complete

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growth medium (2) and rushed to the tissue culture laboratory. There they were chopped into small pieces 2–3 mm^2 and incubated in Balanced Salt Solution containing 0.25% trypsin and 10 mg/ml collagenase for 30 min at 37°C. The pieces (one per flask) were then plated in gridded tissue culture flasks (Lux) with a 25 cm^2 growth area, in 2 ml growth medium. The cultures were incubated at 37°C in an atmosphere of humidified CO_2 in air for 2 days prior to treatment. This allowed explants to attach firmly to the flask before being disturbed and allowed elimination of any explants giving no growth or not attaching. Cis- and carboplatin were diluted appropriately with culture medium so that the required concentration could be added in a volume of 0.05–1.0 ml to each flask. Irradiation was performed either 24 h after or immediately after addition of cytotoxic drugs using a cobalt-60 teletherapy unit delivering 1 Gy/min at 60 cm SSD.

Cultures were incubated without medium change for a further 2 weeks at 37°C and then fixed and stained. The drugs remained in the culture medium for the entire period because of the adverse effects of medium changes on growth of epithelial cells (2). The degree of growth inhibition was assessed by measuring the total area of growth in each flask and expressing the area as a percentage of the untreated control growth ± standard error of the mean (SEM). At least 6 flasks were measured per experimental point and experiments were repeated 3 times using tissue from different patients. The success rate for culture of explants was in the region of 90%. Within sample variation was reduced by screening samples on Day 2 and eliminating any which had not attached. The mean variation on control samples was in the region of ±15% (range ±6–20%). The average outgrowth area for controls after 2 weeks was 160 mm^2 ±34 (SEM) for normal tissues and 138 mm^2 ±32 for tumour tissues, based on the pooled results of 3 experiments, performed on three different patients.

Autoradiography. Cultures were labelled one week after explantation with ^3H thymidine (370 kBq/ml) for 1 h, after which the cultures were carefully washed before being coated with liquid emulsion (Ilford) and stored for 2 weeks to allow development of grains.

Demonstration of cytokeratins. Cultures were fixed in situ on plastic flasks using a solution of 50% acetone in water and air dried. They were rehydrated before staining by three washings in phosphate buffered saline (PBS) and 20 µl of a general mouse antihuman-cytokeratin antibody (Anti-Type II from Amersham International) was added to the culture and incubated for 40–45 min at room temperature in a humidified chamber. After incubation the slides were washed well in PBS. The second antibody, also applied for 40–45 min at room temperature, was an anti-mouse immunoglobulin monoclonal labelled with fluorescin. After further washings in PBS the cultures were rinsed briefly in 1 mol/l Tris HCl buffer (pH 9.0) and mounted in Aquamont before viewing with a fluorescent microscope. Positive and negative controls were carried through the whole procedure every time it was done.

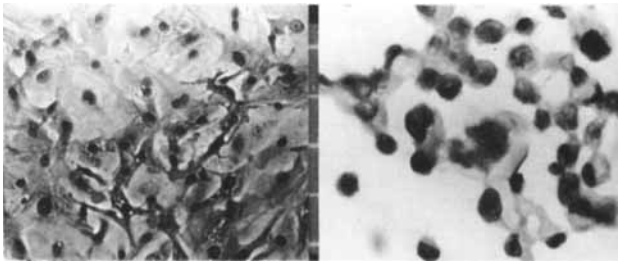

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Fig. 1. Appearance of normal (a) and tumour (b) cells growing from explants after one week in culture.

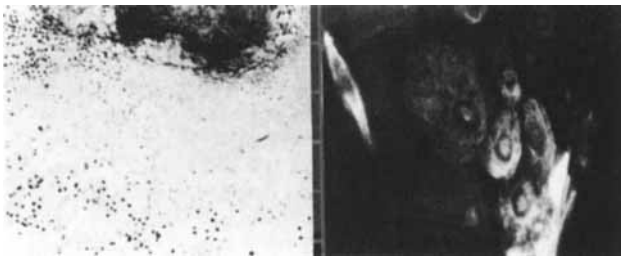

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Fig. 2. a) Autoradiograph of normal bladder cells showing thymidine uptake. b) Fluorescein conjugated anticytokeratin distribution in a culture of normal bladder three weeks after explantation.

Results

Fig. 1 shows a typical outgrowth from a culture of normal (1a) and tumour (1b) urothelium. It is apparent that both normal and tumour cells are morphologically epithelial and that the tumour cells are small, widely spaced and with little cytoplasm, an appearance typical of bladder carcinoma cells.

Fig. 2a shows typical results from an autoradiography experiment. Labelling of cells, particularly at the edge of the explant outgrowth, is evident. In Fig. 2b the epithelial nature of these cells is indicated by the heavy fluorescence of the cells which have been labelled with anticytokeratin. Outgrowths were also checked for the presence of stromal cells using anti-vimentin antibody (Amersham). The outgrowths proved negative for this intermediate filament and, in view of the cytokeratin results, it is concluded that the outgrowths are composed of epithelial cells.

In Fig. 3 data are shown for the effect of increasing concentrations of cis- and carboplatin on growth of normal and tumour bladder urothelium. Levels of drugs were chosen which are equivalent to those in clinical use. Although the drug remained in the medium throughout the 2-week experimental period, platinum analogues break down very fast in plasma and medium (4) and the amount of drug administered initially was, therefore, taken to

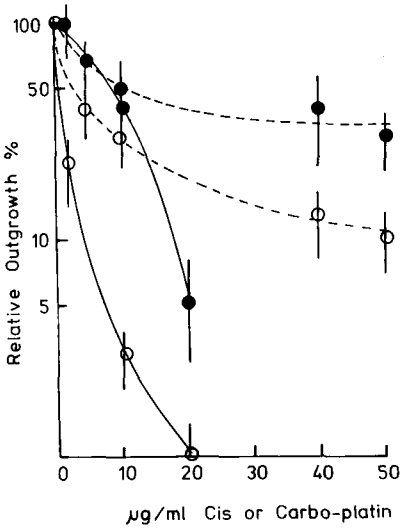

Fig. 3. Dose response curves for normal (○) and tumour (●) bladder explants exposed to increasing doses of cisplatin (——) or carboplatin (---). Error bars are standard errors of the mean.

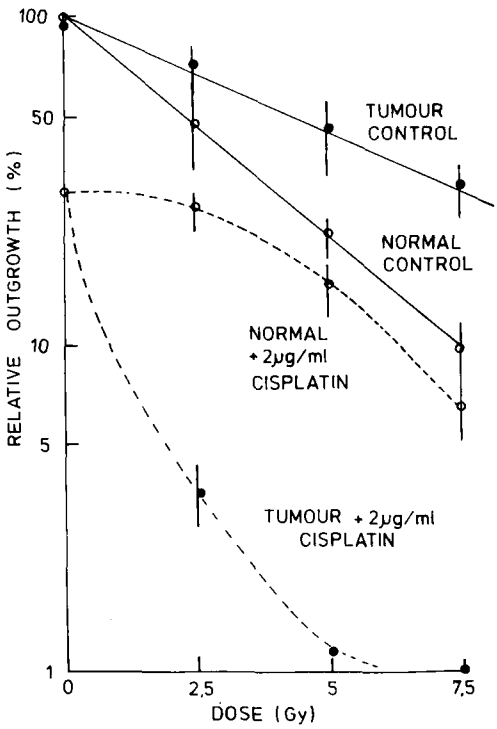

Fig. 4. Effect of 7.5 Gy gamma rays on the dose response curves shown in Fig. 3. Data in Fig. 3 has been normalised to 100% to show the relative effect of radiation in combination with the two drugs. Symbols and error bars as in Fig. 3.

simulate a single dose to a patient. The figure shows that both drugs inhibit cellular growth, cisplatin being more toxic than carboplatin but, as in the case of irradiation (3), normal tissue is considerably more severely affected than tumour tissue.

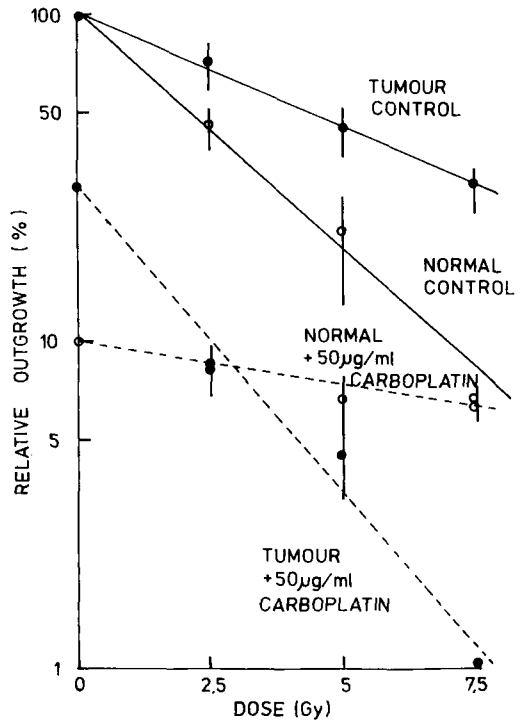

Fig. 5. Dose response curves for irradiated explants pretreated with 2 μg/ml cisplatin (a) or 50 μg/ml carboplatin (b). Symbols and error bars as in Fig. 3.

When the authors used this model system to examine the response of normal and tumour oesophageal tissue to radiation and bleomycin (1), it was found that the tumour cells were only more sensitive to the combined treatment if bleomycin was administered directly before or after radiation, otherwise the tumour proved very radio- and chemoresistant. Since there are clinical reports of successful control of bladder tumours by therapy with radiation and cytotoxic platinum compounds if administered concurrently but not otherwise (5, 6), it was decided to test the effect of these compounds administered immediately before a single dose of radiation (7.5 Gy). The results are shown in Fig. 4 where the data in Fig. 3 has been normalised to 100% to show the relative effect on normal and tumour tissue of exposure to combinations of cis- or carboplatin with radiation. The results are very strange and show that while radiation enhances the effects of platinum analogues on tumour cells, it actually spares normal tissue cells, i.e. the treatment of cisplatin exposed normal cells with radiation causes no additional damage. Fig. 5 (a and b) show the effects of varying the radiation dose while keeping the concentration of cytotoxics added just before irradiation constant. The results indicate that either cytotoxic drug in combination with a low dose of radiation is more effective than if used with a high dose of radiation. Where high doses of both radiation and cytotoxic drug are used, the therapeutic advantage in terms of normal tissue sparing is lost.

Discussion

The technique reported here and in our previous paper (1–3) is being developed as an attempt to provide a method for assessment of chemo- or radiosensitivity of small pieces of human tissue which will not clone reliably in culture. A secondary aim of the work is to enable assessment not only of tumour cell response but also of likely normal tissue damage, so that any favourable or unfavourable therapeutic ratios could be detected and avoided or exploited. This is a problem which has achieved little attention.

A second problem which has received little attention is the development of models which can adequately demonstrate the response of human tissue to therapy. Animal models (reviewed in ref. 7) are unreliable due to life span and size and since the tumours are not very typical of human disease. Available predictive assays, such as the tumour stem cell assay developed by Courtenay & Mills (8), tend to concentrate on the response of clonogenic tumour cells to the proposed therapy. Because of this they give no information on the adverse effects the therapy will have on normal cells which will not clone in soft agar. In addition, it is questionable how relevant the response of the in vitro clonogenic fraction of a tumour, which has been highly processed, is to the actual response of an intact heterogeneous tumour in situ. Evidence of this comes from the reported small or non-existent clonogenic fraction detected in many tumour samples subjected to the soft agar stem cell assay (9) and from the fact that such predictive testing is not used routinely. Weisenthal et al. (10) have discussed these problems in detail and in particular have highlighted the necessity to kill the most resistant cells in a tumour, which are often the non-cycling cells. Those, of course, may subsequently enter the cycling population when the tumour mass is reduced. Their group argued for an assay based on total tumour cell kill and cite high clinical correlations obtained by Glucksmann (11) and Trott (12) as evidence of possible clinical validity of such assays.

Of paticular interest to these authors was assessment of differential response of normal and tumour cells, a factor which we believe is of major importance in therapy, paticularly where radiotherapy forms part of the treatment and which cannot be achieved using the conventional clonogenic assays of most normal epithelial cells (13, 14).

Tumour stem cell assays (15, 16) are reported to give information about clonogenic fractions in tumours but, in addition to the technical problems with these assays cited above, it has been reported (17, 18) that stromal cells contained in tumour specimens can grow with equal efficiency to tumour stem cells. These assays also generally fail to give information about normal epithelial cells from the tumour-bearing organ, since normal cells do not generally clone in agar (19).

In the present paper an in vitro explant model is used to try to predict the response of human normal and tumour bladder tissue to radiation and cytotoxic agents, both singly and in combination. The results for radiation alone confirm those published previously by this group (3) and show that the dose response obtained for normal bladder is in the range usually observed for mammalian cells using clonogenic assays (20), although tumour explants proved to be very radioresistant. It was possible to measure a dose response with both the platinum compounds and again tumour explants were more resistant than normal explants. The results obtained where cytotoxic agents were added to bladder cultures just before irradiation were very similar to those observed for the treatment of oesophageal explants with radiation and bleomycin (1). In particular, the reversal of the therapeutic ratio in favour of normal tissue sparing is repeated, meaning in effect that cis- or carboplatin administered just before radiation act as radiosensitisers for tumour cells but have no effect on or may even protect normal cells.

It is difficult to postulate a mechanism for this effect due to the lack of precise information on the biochemical response of the cell to platinum administration (21). One possible mechanism could be differences either in total glutathione content of the normal and tumour bladder tissues in culture or in the kinetics of glutathione metabolism following exposure to platinum compounds or radiation. Leyland-Jones et al. (22) showed that a transitory increase in glutathione content of 50% could be detected in cells 20 min after cisplatin administration; the levels were back to normal after one hour. Glutathione is known to promote repair in oxygenated cells exposed to radiation but to leave hypoxic cells unaffected (23). It has also been reported that induction of thiols, including glutathione, occurs after irradiation and that the gene amplification responsible persists for several generations (24), so differences in cellular glutathione induction or depletion or in target cell oxygen content could account for the odd results obtained for normal tissue explants (Figs 4 and 5) which, compared to tumour explants, show apparent radioresistance when treated with cisplatin.

Another related mechanism could concern differences in the nature and frequency of induction, or ease of repair, of the DNA lesions induced by radiation or platinum. DNA strand breaks are a predominant lesion induced by radiation (25) while intra strand cross-links between adjacent guanine residues are induced at high frequency by cisplatin (26–28). Vrana & Brabec (29) have reported rapid fixation of radiation induced strand breaks in cells treated by platinum complexes, showing that the conformational change in DNA induced by the platinum compounds made repair of the radiation damage difficult. Speed and efficiency of repair processes would obviously be important in determining the final level of damage. The literature concerning the effects of platinum complexes and radiation on cells is very confusing and could indicate that the cellular response varies due to unidentified factors.

Von der Maase (30) in a review article cites several references showing that cisplatin is a radiosensitiser in *in vitro* studies on bacterial systems and mammalian cell lines and in sòlid tumours, although in other experiments Von der Maase & Overgaard (31) found no sensitisation and state that increases in cell kill following treatment with cisplatin and radiation using experimental bladder cancer models probably could not be attributed to a radiosensitising effect. Douple & Teicher (32) found that platinum linked to radiosensitisers totally inhibited PLD repair in hypoxic cells, while the radioprotector WR 2721 blocked nephrotoxicity induced by cisplatin in rodents without blocking the anti-tumour effects of the drug (33). Obviously there is a degree of confusion about the possible interactions of platinum analogues and radiation but it is clear that tumour and normal cell response can differ.

There is now clinical evidence that cisplatin in combination with radiation is an effective treatment for bladder carcinoma. Two groups in the USA—Shipley et al. (6) of the National Bladder Cancer Group and Coppin et al. (5)—have obtained favourable therapeutic results by treating patients with cisplatin concurrently or followed immediately by radiotherapy.

Validation of a new model or technique of the type described in this paper can only come from carefully designed clinical trials, where the results of the *in vitro* test are applied to the treatment of patients and their response assessed. Obviously the work reported here is at too early a stage of development for such clinical studies to be undertaken. It is useful, however, to attempt to develop an *in vitro* method which overcomes at least some of the theoretical objections to established techniques and which when fully characterised will be amenable in a practical sense to testing in the clinic.

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