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Intravesical Chemotherapy: In Vitro Studies on the Relationship Between Dose and Cytotoxicity

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Summary. The relative importance of two variables, drug concentration and period of exposure, in relation to the therapeutic potential of intravesical chemotherapy was examined in an experimental system. A human bladder cancer cell line was exposed to a range of concentrations of the four drugs commonly used to treat superficial bladder cancer (adriamycin, epodyl, mitomycin-c, thiotepa) for periods of 30, 60 and 120 min. An exponential relationship was observed between clonogenic cell kill and both drug concentration and period of exposure. Thus, under the experimental conditions employed, cytotoxicity is proportional to dose (i.e. concentration x period of exposure). These two variables are of equal importance in relation to tumor cell kill, indicating that maximum therapeutic benefit may be obtained by using the highest concentration achievable for as long as the patient can retain the instillate, bearing in mind the potential increase in toxicity to the patient and the cost.

Key Words: Intravesical chemotherapy, In vitro cytotoxicity, Drug dose.

Introduction

Intravesical chemotherapy is used in the treatment of superficial bladder cancer, either as an adjuvant to surgery to delay tumour recurrence, or to control widespread disease not manageable by surgical methods. However, the conditions under which optimum therapeutic benefit can be achieved using this form of therapy have not been defined (see reviews [13, 14]). The purpose of this study was to examine the influence of two of the factors which can be controlled in the clinic, namely drug concentration and period of exposure, on the in vitro cytotoxicities of the four drugs commonly used for intravesical chemotherapy (adriamycin, epodyl, mitomycin-c, thiotepa). A continuous cell line, RT112, derived from a human bladder tumour was used for this study. RT112 cells produce typical, moderately-well differentiated transitional cell carcinomas on transplantation to nude mice [9].

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Materials and Methods

The RT112 cell line was derived in 1973 from a moderately-well differentiated transitional cell carcinoma of the bladder in a female patient [8].

Cell Culture

The cells were maintained as monolayer cultures in 25 cm^2 flasks (Nunc, Gibco, Paisley, Scotland), in RPMI 1640 medium (Gibco) supplemented with 5% heat-inactivated foetal calf serum (SeraLab, Crawley Down, England) and 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland). For the purpose of this study, the same batch of serum was used throughout. The cells were kept at 36.5 °C in a humidified atmosphere of 5% CO_2 in air, and used over a restricted range of 10 passages. For subculturing, the cells were detached using 0.05% trypsin (Difco 1: 250; Difco, London, England) and 0.016% versene (ethylenediamine- tetraacetic acid disodium salt, EDTA; BDH Chemicals, Poole, England) and split in a ratio of between 1:5 and 1:10. Mycoplasma was not detected using aceto-orcein stained monolayers [5], or in nutrient agar culture [1].

Colony Forming Assays

A single-cell suspension of exponentially-growing RT112 cells was plated at a density of 12,800/well in a 96-well microtest plate (Nunc) and incubated for 48 h at 36.5 °C in a humidified atmosphere of 5% CO_2 in air to permit the cells to resume an exponential growth rate. Then the medium was removed and replaced with either fresh medium (5 control wells) or medium containing a range of drug concentrations (3 wells per drug concentration). After a 30, 60 or 120 min exposure, the medium was removed and the cells washed

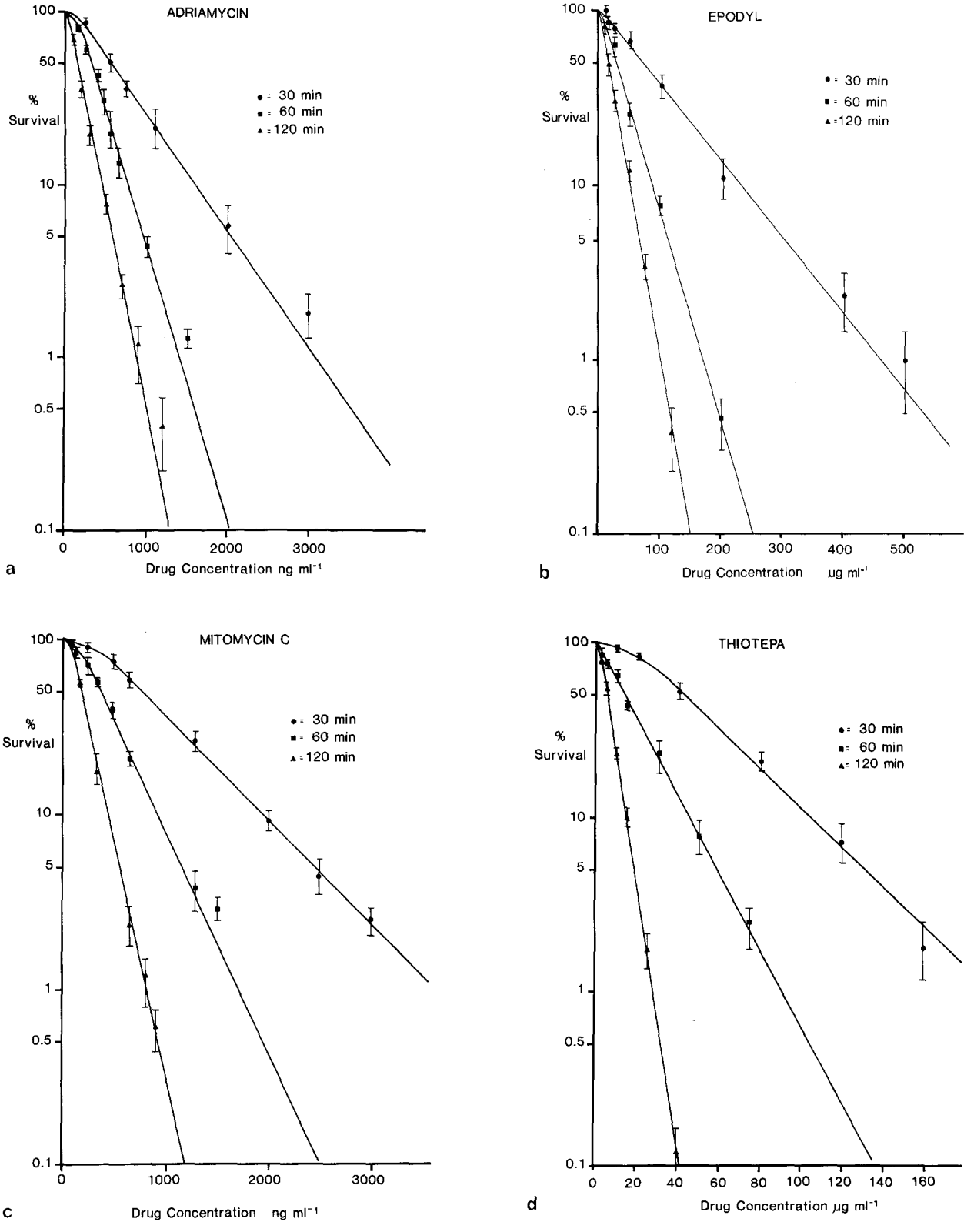

Fig. 1. Dose response curves of RT112 cells to adriamycin (a), epodyl (b), mitomycin-c (c) and thiotepa (d). Clonogenic cell survival on a logarithmic scale for each exposure period (30, 60 and 120 min) is plotted against drug concentration on a linear scale. The bars indicate one standard error either side of the mean

Table 1. Drug concentration (μg/ml) required to kill 90% of the clonogenic cells (ID90) following exposure periods of 30, 60 and 120 min

	Period of exposure (min)		
	30	60	120
Adriamycin	1.60	0.78	0.45
Epodyl	235	90	50
Mitomycin C	1.96	0.92	0.42
Thiotepa	106	45	15

using three aliquots of 0.2 ml medium and once with 0.2 ml calcium and magnesium free phosphate buffered saline (PBSA) [2]. The cells were then detached using 0.2 ml of enzyme solution (0.01% trypsin and 0.003% versene in PBSA) and diluted as necessary in medium to produce cell densities yielding 100–200 colonies when 0.2 ml cell suspension was added to 5 ml prewarmed and gassed medium in a 5 cm petri dish (Nunc). The medium was replenished after 7 days, and after 14 days incubation the colonies were fixed in methanol (BDH), and stained with 10% Giemsa (BDH). Colonies consisting of 50 or more cells were counted using a binocular dissecting microscope. The mean number of colonies per drug concentration was expressed as a percentage of the control value. The data are derived from a minimum of 3 experiments for each drug at each exposure time.

Drugs

All drugs were made up immediately prior to use in PBSA, with a final dilution in medium. Standard pharmaceutical preparations of adriamycin (doxorubicin HCl; Farmitalia Carlo-Erba, Barnet, Herts, England), epodyl (ethoglucid; Imperial Chemical Industries, Macclesfield, Cheshire, England), mitomycin-c (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) and thiotepa (triethylenethiophosphoramide; Lederle Laboratories, Gosport, Hants, England) were used.

Results

The survival of RT112 cells following exposure to a range of concentrations of the four drugs during exposure periods of 30, 60 and 120 min are shown in Fig. 1a–d. Clonogenic cell survival is plotted on a logarithmic scale against drug concentration on a linear scale. An exponential decrease in cell survival was observed in response to increasing concentrations of epodyl and, following a short "shoulder" region at low drug concentrations, similar dose-response curves were obtained with adriamycin, mitomycin-c and thiotepa. It can be seen that there is also an approximately logarithmic relationship between the period of exposure and clonogenic cell survival.

The drug concentrations required to reduce clonogenic cell survival by 90% (ID90) were derived from Fig. 1 for each drug and exposure period and are listed in Table 1.

Discussion

The therapeutic value of intravesical chemotherapy for superficial bladder cancer is well documented [13, 14]. Intravesical instillation has two major advantages – it directly exposes the urothelium to a relatively high drug concentration and, because uptake is limited, causes minimal systemic toxicity. However, optimum therapeutic conditions have not been defined [13, 14]. This study demonstrates that two of the varaibles under the clinicians' control, drug concentration and period of exposure, are equally important in relationship to in vitro tumour cell kill.

The results indicate that within the limits of the experimental conditions used there is a logarithmic relationship between clonogenic cell kill and both drug concentration and period of exposure. The relationship can be expressed mathematically [3] using the equation: surviving cell fraction = e^{-ktc} (where e = basis natural logarithm, k = constant related to the sensitivity of the cells, t = duration of exposure and c = drug concentration). Thus, if the drug concentration remains constant during the period of treatment, clonogenic cell kill is proportional to the concentration times the exposure period, i.e. the drug dose. These data relating cytotoxicity to dose are consistent with previous studies in a variety of mammalian cell systems, including human bladder cancer cells in vitro [11]. However, this direct relationship between cytotoxicity and dose will only hold under certain well-defined conditions. For example, the presence of a resistant cell population will either result in a biphasic or a shallow dose-response curve, in which case increasing dose may have little effect on response. In addition, features such as pH, drug stability and metabolism can alter drug activity during the exposure period, making interpretation of data more complex (for discussion see [18]). Another exception are cell-cycle stage specific drugs, such as methotrexate, which do not produce exponential dose-response curves during limited exposure periods [10] and therefore for these drugs the relationship will not apply. While these points should be considered, the four drugs used in this study are relatively stable, and the relationship between tumour cell kill and dose is likely to be valid for those tumours which are sensitive to chemotherapy.

To what extent are these observations relevant to clinical practice? In this model system and in patients receiving intravesical chemotherapy, urothelial tumour cells are exposed to a predetermined drug concentration for a known period of exposure. The major differences are that in the bladder the effective concentration of the drug will be altered by systemic uptake and dilution by urine, and there may be differences in drug stability, metabolism and pH. In addition, the concentration to which individual tumour cells are exposed will depend on the degree of penetration of the drug and the depth of the tumour cells, which may explain why much lower drug concentrations are required to kill cell monolayers in vitro. Bearing in mind the importance of these considerations, the relationship between tumour cell kill and drug dose observed in vitro is still

likely to apply in the clinical situation. In support of this hypothesis, in nearly all clinical and experimental animal studies using effective therapy, response rates have been consistently and markedly dose-related [6, 16]. Thus, there are three implications for the routine use of intravesical chemotherapy. Firstly, the drug should be used at the highest concentration compatible with lack of toxicity to the patient and reasonable cost. Secondly, the drug should be retained in the bladder for as long as possible, unless there is a limiting increase in toxicity to the patient. Thirdly, it should be appreciated that a doubling of the exposure period may be equivalent to a doubling of the drug concentration in terms of tumour cell kill.

Some authors have suggested that drug exposure of human bladder cancer cells as a single cell suspension or monolayer might be used for predicting the response of individual tumours [15, 17], or preclinical screening [7, 11, 12]. However, in all these studies thiotepa was less cytotoxic in comparison with other drugs than would be expected on the basis of its proven clinical value. These findings highlight a limitation of such two-dimensional model systems, which do not take into account the degree of drug penetration into tumours. Thiotepa, in contrast to epodyl, adriamycin and mitomycin-c, penetrates the bladder wall and enters the circulation in significant amounts [13]. Consequently, the tumour cells furthest from the lumen may be exposed to relatively high concentrations of thiotepa, perhaps explaining the apparent clinical effectiveness of this drug. Using spheroids, an in vitro three-dimensional model system, developed from a human bladder cancer cell line, it was shown that adriamycin penetration, and consequently cytotoxicity, was reduced in the inner cell layers [4]. Therefore, to make useful predictions concerning the comparative value of drugs for intravesical chemotherapy it will be necessary to use both two- and three-dimensional model systems. Useful drugs are likely to have a low molecular weight (to facilitate tumour penetration), have a relatively high degree of cytotoxicity in two-dimensional systems, and be able to penetrate and kill cells towards the centre of three-dimensional systems.

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