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Cytotoxicity of 5-fluorouracil released from a bioadhesive patch into uterine cervical tissue

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Abstract

HeLa cells were used as a model cell line to evaluate the cytotoxic concentration of 5-fluorouracil as a candidate drug for the topical treatment of cervical intraepithelial neoplasia (CIN). Cytotoxicity was measured by exposing cell suspensions to increasing concentrations of drug and measuring the decreased rate of cell growth. Results were confirmed by photographing monolayers and estimating the ratio of cells entering mitosis. A drug concentration of 10^{-4} M was found to be cytotoxic. Cervical tissue samples were exposed for either 4 or 24 h periods to 5-fluorouracil released from a bioadhesive cervical patch containing 20 mg of drug. The concentration distribution of 5-fluorouracil through cervical tissue were estimated from the amounts, as determined by HPLC, extracted from tissue slices harvested at depths down to 5 mm from the surface. Even at this depth, the tissue concentration following a 24-h exposure to 5-fluorouracil was 100-fold that of the determined cytotoxic drug concentration, indicating that the patch delivery system could result in clinically effective drug concentrations in those areas of the cervical stroma where pre-cancerous lesions characteristic of cervical intraepithelial neoplasia can occur. © 1997 Elsevier Science B.V.

Keywords: HeLa cells; Cytotoxicity; 5-Fluorouracil; Cervical intraepithelial neoplasia; Bioadhesive

1. Introduction

Cervical intraepithelial neoplasia (CIN) is a common and potentially malignant condition affecting women from a wide age group. The condition is initially localised in the squamous epithelium, which is composed of five histologically distinct layers (Fluhman, 1961) and lines the portion of the cervix uteri exposed to the vaginal environment. As CIN progresses, a greater thick-

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ness of this layer becomes occupied, until the full thickness is replaced by undifferentiated neoplastic cells. This stage is described as a carcinoma in situ (CIS) and often progresses to an invasive cervical carcinoma (ICC).

CIN I and CIN II, denoting mild and moderate CIN, can be treated as an out-patient procedure. Generally, ablative techniques, such as cold coagulation and laser therapy, are the methods of choice. If the disease has progressed to CIN III or CIS, then excisional techniques, such as cone biopsy and large loop excision of the transformation zone (LLETZ) are used. However, ablative and excisional techniques will both alter the cervical architecture. Thus, the shape and extent of tissue ablated from the cervical surface will determine the post-operative anatomy of the cervix (Beresford et al., 1990) and this may have implications for future fertility. Significantly, some loci of CIN will reside in crypts which are often 2–3 mm below the colposcopically observable surface (Anderson and Hartley, 1980). Therefore, there is a risk that cancerous loci will be missed during treatment and future reoccurrence of CIN will arise.

The location of the cervix is unique in allowing ready inspection and topical treatment. Therefore, it is an ideal candidate for topically-administered cytotoxic therapy. Additionally, its mucosal epithelial surface allows a patch constructed from mucoadhesive polymers to be located for extended periods of time. We have previously described the construction of a 5-fluorouracil-loaded patch and profiled the delivery of drug through cervical tissue (Woolfson et al., 1994). However, for the patch concept to be successful, it is essential to know at what concentration 5-fluorouracil will begin to exert its cytotoxic effect and if that concentration can be achieved at a depth where cancerous loci may be expected to reside. Thus, this study determines the minimum cytotoxic concentration of 5-fluorouracil against HeLa cells and relates this to concentrations of the drug found at various depths in cervical tissue following in vitro application of a bioadhesive patch delivery system.

2. Materials and methods

2.1. Materials

HeLa cell line ECACC No. 85060701 was obtained from the European Collection of Animal Cultures, PHLS Centre for Applied Microbiology and Research (Porton Down, Salisbury, Wilts.). 5-Fluorouracil was obtained from David Bull Laboratories (Mulgrave, Victoria). Giemsa's stain, Gurr's improved R66, was purchased from BDH chemicals (Poole, Dorset) and lyophilized trypsin, 0.1% working solution, from Gibco Life Technologies Ltd. (Paisley, Scotland).

2.2. Cytotoxic challenge and visual examination of HeLa monolayers

A stock suspension of HeLa cells was grown in medium and subcultured into 12 flasks. Each flask contained approximately 1×10^5 cells in 5 ml medium. An appropriate volume of 5-fluorouracil solution (0.5 ml in phosphate-buffered saline, pH 7.2, PBS) was added, giving final concentrations of 10^{-8}–10^{-4} M in successive flasks. Flasks were incubated at 37°C for 24 h in a 5% CO_2 atmosphere. After incubation, flasks were washed with sterile phosphate-buffered saline (pH 7.2) and methanol (5 ml) was added for 10 min to fix the cells. The methanolic solution was then discarded and the film allowed to dry for several minutes. The cells were stained with Giemsa's stain for 5 min, allowed to dry and counted visually.

2.3. Cytotoxic challenge and counting of HeLa suspensions

A stock suspension of HeLa cells was grown until a confluent monolayer was obtained. Cells were dislodged from the culture flask with 25 min incubation in trypsin solution. From the resultant cell suspension, 0.5 ml was inoculated into 12 flasks, each containing 5 ml medium. The flasks were grown for 24 h to allow the monolayer to re-establish. Each flask was then incubated for a further 24 h in media containing from 10^{-8}–10^{-4} M 5-fluorouracil, successively. The exposed

monolayer was lifted from the plastic dish using trypsin and dispersed using gentle shearing through the pipette tip against the flask wall. Monodispersion of the cells was confirmed by microscopic examination and, if incomplete, shearing was repeated. The cell suspension was counted on a Coulter counter.

2.4. Exposure of cervical tissue to 5-fluorouracil

Excised cervical tissue (from hysterectomies, donated with permission in all cases) was cut into slabs, 1 cm^2 across the epithelial face and approximately 10 mm deep. The tissue was clinically judged to be healthy, the donors being free of cervical disease, and was visually checked for surface damage before use. The tissue was supported on a stainless steel filter grid (Millipore Corp., Cambridge, MA) placed across the top of the reservoir of a Franz cell, as previously described (Woolfson et al., 1994). The reservoir was filled with 10.0 ml of sterile phosphate buffered saline (pH 7.2), sufficient to bring the fluid level up to the grid and expel traces of air.

A bioadhesive cervical patch containing a total of 20 mg of 5-fluorouracil was prepared as previously described (Woolfson et al., 1994). The patch was applied to the uppermost epithelial layer of the tissue slab such that the bioadhesive layer and tissue were in direct, intimate contact. Patches were applied for fixed time periods of either 4 or 24 h. The patch and tissue were separated when the required penetration time had elapsed. The tissue was then flash-frozen by exposing it to a liquid nitrogen atmosphere, without immersion, for 3 min.

The tissue block was bisected perpendicularly to the drug flux and one half mounted on the stage of a cryostatic microtome, as previously described (Woolfson et al., 1994). Groups of ten consecutive slices were taken and placed in preweighed vials. These slices were cut perpendicular to the plane of drug flux and parallel to the epithelium. Slices were cut at a microtome setting of 50 μm and stored in a liquid nitrogen atmosphere until use.

2.5. Determination of 5-fluorouracil in cervical tissue slices

The contents of one vial (10 tissue slices) were dissolved in 1M NaOH (2 ml) with ultrasonification (2 h). HCl (1.5 ml, 2 M) was added to the resulting cloudy solution (1 ml) which was then allowed to settle for 24 h. The supernatant was withdrawn, filtered and the filtrate (1 ml) added to aqueous thymine solution (0.2 ml, 40 $\mu g\ ml^{-1}$). The resulting solution was agitated with extraction solvent (5 ml, 20% *n*-propanol/ether) and allowed to settle for 1 h. A sample (2 ml) was then withdrawn and the solvent removed under compressed air. The residue was reconstituted with water (1 ml) and the concentration of 5-fluorouracil determined by high performance liquid chromatography (HPLC).

The HPLC system consisted of a Gilson Model model 302 pump, LKB model 2151 UV detector, Waters model 710B autoinjector and a Hewlett-Packard model 3390A integrator. Separations were carried out on a stainless steel chromatographic column (250 × 4.6 cm I.D.) packed with Spherisorb ODS 5 μm. The mobile phase was methanol/water (1:10) at a flow rate of 1 ml min^{-1}, with detection at 267 nm, injection volume 20 μl and an analysis run time of 10 min. The internal standard was thymine (40 $\mu g\ ml^{-1}$, 0.2 ml per 1 ml of the tissue extract). Quantitation was obtained by determining peak area ratios of 5-fluorouracil to thymine and comparing these to standard injections of 5-fluorouracil, similarly extracted. Linear calibrations were obtained from 1–100 $\mu g\ ml^{-1}$ ($r = 0.999$).

3. Results and discussion

Exposure to 5-fluorouracil reduced the ability of HeLa cells to undergo mitosis in vitro. This could be observed directly by examining microscopically pre-exposed and stained monolayers. Mitotic figures are readily identifiable as a darkly staining body resembling a figure '8'. An estimate for the mitotic activity was obtained by determining the number of mitotic bodies in the field of view per 100 cells counted (Table 1). Mitotic

Table 1
Detection of mitotic cells in the monolayer

5-Fluorouracil concentration (M)	Observable mitotic cells/100 cells counted
Blank	5.17
10^{-8}	5.22
10^{-7}	4.71
10^{-6}	3.42
10^{-5}	0.64
10^{-4}	0.00

Count is expressed as a ratio of mitotic cells per 100 cells counted

bodies were readily located across stained monolayers exposed previously to a blank medium containing no 5-fluorouracil, and to media containing 10^{-8} and 10^{-7} M 5-fluorouracil, respectively. However, flasks containing 10^{-5} M 5-fluorouracil showed a marked decrease in cell activity and those containing 10^{-4} M showed no evidence of mitosis.

A further quantitative estimation of the survival of HeLa cells was performed by looking at the growth in cell numbers during exposure to increasing 5-fluorouracil concentrations in the growth medium. As shown in Fig. 1, the growth in population after 24 h decreased only slightly, until media containing 10^{-5} M 5-fluorouracil were used. There was a sharp decrease in cell growth, seen more profoundly in media containing 10^{-4} M 5-fluorouracil. These results clearly show that cell growth is inhibited at 10^{-4} M 5-fluorouracil.

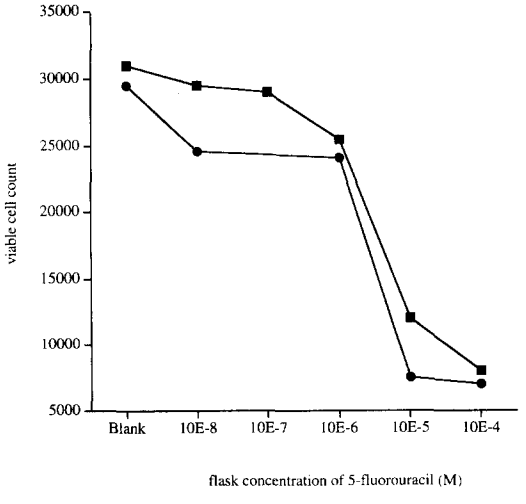

Fig. 1. Viable HeLa cell counts in two series of flasks, each exposed to identical concentrations of 5-fluorouracil.

Ideally, it would have been desirable to grow cultures of pure cervical epithelial cells (Pichart, 1964) for this study. However, HeLa cells were chosen because of the availability, ruggedness and widespread use of this particlar cell line. HeLa is an undifferentiated cell line originally derived from a uterine carcinoma cell line. Results from population growths and visual examination clearly show that 5-fluorouracil arrests cell division at a concentration of 10^{-4} M. This agrees with work by Eaglstein et al. (1970), who used intradermally injected tritiated deoxyuridine to demonstrate that 5-fluorouracil blocked DNA synthesis in both normal and actinic keratosis. More importantly, it was shown that a minimum tissue concentration of 10^{-5} to 10^{-4} M 5-fluorouracil was required to inhibit thymidylate synthetase, the target enzyme for 5-fluorouracil.

5-Fluorouracil has been shown to be active against a variety of solid tumours, including those in breast, colon, rectum and cervix (Young et al., 1960; Ansfield et al., 1962). It is, therefore, an ideal drug to incorporate within a bioadhesive patch intended to deliver locally 5-fluorouracil to the uterine cervix. We have previously described the drug release characteristics of this patch (Woolfson et al., 1995a). It is also an essential requirement for this type of patch-based system that the cytotoxic drug can be delivered to a depth of approximately 4 mm from the cervical surface, which is needed to arrest cancerous loci that reside at this level and which are not apparent on visual examination of the cervical surface. Thus, cervical tissue blocks were exposed in vitro for either 4 or 24 h to bioadhesive patches containing 20 mg of 5-fluorouracil. Drug release from the patch resulted in passive diffusion into the tissue along a concentration gradient, the qualitative distribution of the drug through cervical tissue having been previously established by autoradiography (Woolfson et al., 1995b). In the present study, the amount of 5-fluorouracil was determined by HPLC analysis of extracts from

Table 2
Amounts and estimated concentrations of 5-fluorouracil found at various depths into cervical tissue following application of a bioadhesive patch containing 20 mg of 5-fluorouracil for 4 and 24 h, respectively

Tissue depth (mm)	5-Fluorouracil			
	Amount found in tissue ($\mu g\ mg^{-1} \pm$ S.D., $n = 4$)		Estimated tissue concentration (M)	
	4 h Exposure	24 h Exposure	4 h Exposure	24 h Exposure
0.5	1.60 ± 0.13	—	1.23×10^{-2}	—
1.0	1.57 ± 0.16	2.51 ± 0.17	1.21×10^{-2}	1.93×10^{-2}
1.5	1.10 ± 0.09	2.30 ± 0.15	8.40×10^{-3}	1.77×10^{-2}
2.0	0.91 ± 0.09	2.22 ± 0.11	7.00×10^{-3}	1.71×10^{-2}
2.5	0.89 ± 0.05	1.93 ± 0.13	6.85×10^{-3}	1.49×10^{-2}
3.0	0.66 ± 0.03	—	5.08×10^{-3}	1.25×10^{-2}
3.5	0.52 ± 0.04	1.62 ± 0.17	4.00×10^{-3}	1.16×10^{-2}
4.0	0.48 ± 0.07	1.51 ± 0.10	3.69×10^{-3}	—
4.5	0.47 ± 0.11	—	3.62×10^{-3}	1.07×10^{-2}
5.0	0.23 ± 0.04	1.39 ± 0.12	1.77×10^{-3}	1.02×10^{-2}

groups of ten tissue slices harvested at specific depths into the cervical tissue block following drug exposure. Results are presented in Table 2.

A direct comparison can now be drawn between drug levels achievable after systemic therapy and localised targeted therapy from a patch. Myers et al. (1976) stated that a concentration of 10^{-4} M 5-fluorouracil was achievable after intravenous dosing, but administration of a relatively high dose of 15 mg kg^{-1} was required. On this basis, for an adult of 100 kg body weight, a dose of 1500 mg would be necessary. By comparison, if the reasonable assumption is made that the density of cervical tissue approximates to that of water, it is possible to estimate the concentrations of 5-fluorouracil present in the cervical tissue samples at various depths (Table 2), following local topical application of the drug. Given that the cytotoxic concentration of the drug against HeLa cells was determined as 10^{-4} M, it is readily apparent that this cytotoxic concentration was exceeded at all depths up to 5 mm for both 4 and 24 h applications. Thus, after 24 h exposure to the drug, the cytotoxic concentration was exceeded by a factor of approximately 200 at the tissue surface and, even at a depth of 5 mm into the tissue, the estimated concentration of 5-fluorouracil was approximately 100 greater than the cytotoxic threshold. However, systemic levels of 5-fluorouracil were nevertheless small, as confirmed by a lack of reported side effects during patch use (Sidhu et al., 1997), due to the bilayer design of the patch. This study therefore demonstrates that it is possible to achieve cytotoxic concentrations of 5-fluoruracil in cervical tissue following topical application and that such concentrations are present at sufficient depth to be of clinical significance in cervical intraepithelial neoplasia. Although ablative and excisional methods are highly effective in this condition, they do require specialised equipment and facilities. Development of the cytotoxic patch system may therefore be particularly relevant in third world countries, where such facilities are not always readily available.

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