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Endocytosis of proteins by kidney tubule cells: inhibition by the aminoglycoside gentamicin

Nephrotoxicity is a major limitation to the long term use of aminoglycoside antibiotics such as gentamicin. This nephrotoxicity is associated with damage to both kidney glomerulus and tubule and is dependent on such factors as dose, duration of exposure, age and sex [1]. Early renal dysfunction has been related to the accumulation of aminoglycosides in the lysosomes of proximal tubule cells [2, 3]. Gentamicin also accumulates in lysosomes of other cell types such as fibroblasts [4] as has been shown for other weak bases [5].

Proximal tubule cells in the kidney are responsible for the reabsorption and degradation of protein remaining after ultrafiltration in the glomerulus [6]. It has been suggested [7] that an initial event in normal tubular protein reabsorption is an interaction between the protein and an anionic binding site at the proximal tubule brush border. Additionally, competition for such sites by polycationic aminoglycosides appears to take place [8]. Thus the effect of gentamicin on the renal handling of proteins is of interest.

We have recently used a kidney cell line—Madin Darby Canine Kidney (MDCK) cells—to study the selectivity in adsorptive endocytosis of different size proteins by tubular cells [9]. The present study utilises this cell line to investigate the effects of gentamicin on the uptake and degradation of rat plasma proteins.

Materials and methods

MDCK cells were maintained in monolayer culture in Eagles minimum essential medium (MEM) (Flow Laboratories) supplemented with 10% heat inactivated foetal calf serum and antibiotics. Rat serum (Miles Laboratories, Bucks, U.K.) was inactivated by heating at 56° for 30 min and then separated into three molecular size fractions by gel filtration on a Sepharose 6B column (Pharmacia, Sweden). Gel electrophoresis on 7.5% non-denaturing gel in Tris–glycine pH 8.9 showed the largest molecular size fraction to contain predominantly macroglobulins. The intermediate size fraction comprised gammaglobulins with some albumin and the smallest molecular size fraction contained albumin and the smaller proteins. Aliquots of concentrated fractions were iodinated using the iodogen method [10] and the reaction products dialysed against phosphate buffered saline (PBS) after which less than 2% of the radioactivity was soluble in 5% trichloroacetic acid (TCA). Competition studies (data not show) indicated that the iodinated protein was recognised in a similar manner to the unlabelled protein as would be expected with the mild iodination procedure employed. All reagents were of the best available commercial grade.

MDCK cells were seeded at low density and allowed to grow to confluence over a period of 5 days in the presence of gentamicin (Flow Laboratories) in the concentration range 250–1000 μg/ml of culture medium. For the measurement of endocytosis, [^{3}H]-sucrose (Amersham International) or ^{125}I-labelled proteins were then presented to the resultant confluent cell monolayers in fresh serum supplemented culture medium containing gentamicin such that gentamicin was present throughout the experimental period at the same concentrations as used in the preincubations. At the commencement of uptake the confluent monolayer consisted of viable cells and viability was maintained throughout the experiments as judged by morphology and retention of cell protein in the monolayer.

In order to measure uptake and degradation at chosen times after addition of the substrate, the media were harvested and the cells washed four times with ice-cold PBS to remove extracellular radioactivity. The cells were then lysed by the addition of 0.1% Triton X-100 in PBS. To measure uptake and degradation, media and cell fractions were precipitated with 5% TCA and TCA soluble and insoluble fractions counted separately by a gamma counter (Ultragamma 1280, LKB) or, for [^{3}H]-sucrose uptake, aliquots counted directly in a liquid scintillation counter (Packard Tricarb 460C). An aliquot of each cell sample was assayed for protein by the method of Lowry *et al.* [11] using bovine serum albumin as standard. Uptake was

defined as the volume of culture medium (μl) whose contained substrate is captured per mg cell protein and is calculated as {(total TCA soluble + cell TCA insoluble)/ radioactivity supplied per μl medium}, to allow for both material which has been degraded and material undergoing degradation in the cell. Degradation (%) is expressed as (total TCA-soluble radioactivity/total radioactivity) × 100. Student *t*-tests were used to determine significant differences.

Results and discussion

The influence of gentamicin on the uptake of [^{3}H]-sucrose by MDCK cells is shown in Fig. 1a. Sucrose has previously been used as a marker for fluid phase endocytosis [12] and our data are consistent with this for these cells. Incubation of MDCK cells in the presence of 500 μg/ml gentamicin for 5 days caused an increase in fluid phase endocytosis after 24 hr. In contrast, the presence of 1000 μg/ml had a marked inhibitory effect on uptake over the time course of the experiment. Such biphasic effects of amines are now well known [5] and are due to the complex nature of the amine–cell interaction discussed in more detail below.

Growing MDCK cells in the presence of 250–750 μg/ml gentamicin for 5 days had no effect on the uptake and degradation of the largest molecular size plasma protein fraction (Tables 1 and 2). However, at the highest concentration of gentamicin studied (1000 μg/ml) the uptake was reduced to 50% of the control value after 24 hr (Table 1, Fig. 1b). The uptake of the intermediate fraction was reduced in the presence of 500–1000 μg/ml gentamicin to a minimum of 60% of the control value after 24 hr (Table 1). The degradation of this fraction was also reduced after 24 hr to 72%, 54% and 46% after preincubation with 500, 750 and 1000 μg/ml respectively (Table 2).

Similarly, the presence of gentamicin in the concentration range 500–1000 μg/ml decreased the uptake of the smallest plasma protein fraction compared to the controls. After 24 hr, following preincubation with 500 μg/ml gentamicin, the uptake of the smallest plasma protein fraction was reduced to 41% of the control value (Table 1). At this gentamicin concentration, degradation was reduced to 78%.

This interference of gentamicin on the renal handling of proteins by kidney tubule cells is in agreement with results of other workers [13–15] using whole animal systems. The present study examines the effect of gentamicin on the endocytosis of proteins by kidney tubule cells in isolation and thus allows a more detailed examination of the reabsorption of proteins by proximal tubule cells than those using the whole kidney *in vivo*. Cojocel and Hook [13] using a perfused kidney system have demonstrated an almost complete abolition of ^{125}I-lysozyme degradation at a gentamicin perfusate concentration of 0.5 mg/ml. Lysozyme has a molecular weight of 14,400 Da and would thus be contained within our smallest molecular weight fraction. This inhibition of degradation could be due primarily to the inhibition of tubular uptake such as observed here. In addition, the lysosomal accumulation of aminoglycosides has been shown to inhibit the activity of some phospholipases and proteolytic enzymes [3, 16] which may lead to a reduction in renal degradation independent of inhibition of endocytosis. The pH of several intracellular compartments including endosomes and lysosomes are perturbed in the presence of amines [5]; a likely consequence of this could be an inhibition of degradation which may

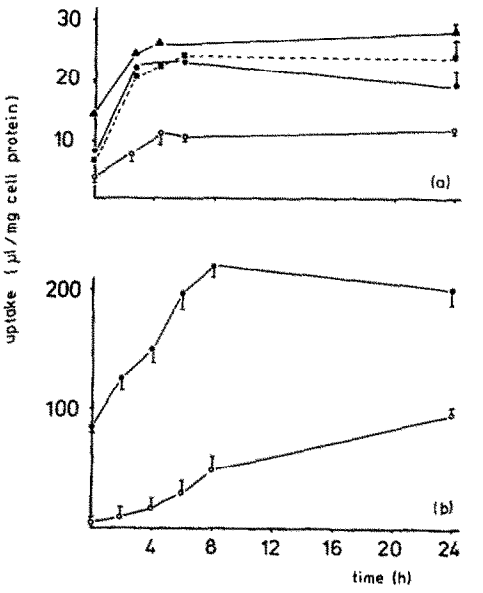

Fig. 1. The effect of gentamicin on the uptake of (a) [^{3}H]-sucrose and (b) the largest size plasma protein fraction by MDCK cells in culture. Cells incubated in MEM containing 10% serum in the absence (control) (●), or in the presence of 500 μg/ml (▲), 750 μg/ml (■) and 1000 μg/ml (○) gentamicin. Each value represents data from 6 replications ± S.D.

Table 1. Effect of gentamicin on the uptake of different size plasma protein fractions by MDCK cells in culture

Gentamicin concentration	Time of incubation	Uptake (μl/mg cell protein) Low mol. wt. fraction	Intermediate mol. wt. fraction	High mol. wt. fraction
None (control)	6	27.5 ± 3	44 ± 3.2	188 ± 16
	24	90 ± 15	96 ± 5.3	201 ± 20
250	6	20 ± 4.0	40 ± 4.0	181 ± 13
	24	74 ± 19	89 ± 6.1	191 ± 18
500	6	18.4 ± 3.3*	35 ± 3.1*	175 ± 13.4
	24	37 ± 5.0*	65 ± 4.2*	196 ± 13
750	6	21 ± 0.9*	25 ± 5.1*	180 ± 20
	24	51 ± 3.5*	59 ± 3.1*	210 ± 17
1000	6	24 ± 2.0	41 ± 6.0	30 ± 8*
	24	49 ± 4.1*	55 ± 4.7*	95 ± 5*

Cells incubated in MEM containing 10% serum plus gentamicin for 5 days prior to experiment. Each value represents data from 4 replications ± SD. *For these values $P < 0.01$.

Table 2. Effect of gentamicin on the degradation of different size plasma protein fractions by MDCK cells in culture

Gentamicin concentration	Time of incubation	Degradation (%) Low mol. wt. fraction	Intermediate mol. wt. fraction	High mol. wt. fraction
None (control)	6	0.4 ± 0.09	1.2 ± 0.09	1.75 ± 0.2
	24	1.6 ± 0.06	3.8 ± 0.2	4.25 ± 0
250	6	0.3 ± 0.15	1.14 ± 0.1	0.85 ± 0.15
	24	1.74 ± 0.37	3.75 ± 0.3	4.13 ± 0.2
500	6	0.1 ± 0.03*	1.98 ± 0.04	1.65 ± 0.3
	24	1.25 ± 0.11*	2.75 ± 0.11*	4.20 ± 0.35
750	6	0.1 ± 0.04*	1.6 ± 0.3	1.7 ± 0.09
	24	1.42 ± 0.1*	2.05 ± 0.2*	4.5 ± 0.5
1000	6	0.13 ± 0.05*	1.09 ± 0.09	0.9 ± 0.2*
	24	1.39 ± 0.1*	1.75 ± 0.1*	1.68 ± 0.2*

Cells incubated in MEM containing 10% serum plus gentamicin for 5 days prior to experiment. Each value represents data from 4 replications ± SD. *For these values $P < 0.01$.

feedback on uptake. However, no clear indication of such an inhibition of degradation was observed in this study. Thus the prime target of gentamicin seems to be endocytosis though the mechanism may be direct or indirect. This inhibition of uptake in tubule cells may well contribute to the proteinuria found in aminoglycoside nephrotoxicity.

In summary, a kidney tubule cell line—MDCK cells—has been used to study the effects of gentamicin on the endocytosis of size fractionated plasma proteins by kidney tubule cells. Cells cultured in the presence of gentamicin for 5 days show reduced uptake and degradation compared to the corresponding control incubation. This inhibition by gentamicin may be attributed to its effect on endocytosis.

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Inositol phospholipid metabolism and platelet function*

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Platelet agonists of physiological or pathophysiological significance include von Willebrand factor, collagen, thrombin, ADP, platelet-activating factor, serotonin, prostaglandin endoperoxides, thromboxane A_2, vasopressin and epinephrine. They all bind to specific receptors on the platelet surface and trigger molecular events which finally lead to the various platelet responses, i.e. adhesion, shape change, release reaction and aggregation.

An early biochemical event upon platelet activation is the degradation of inositol phospholipids by phospholipase C resulting in the rapid formation of inositol trisphosphate, 1,2-diacylglycerol, and its phosphorylated product, phosphatidic acid [1–3]. These substances remain inside the platelets and act as mediators and/or modulators of platelet

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