

Nephrotoxicity Testing *in Vitro*—What We Know and What We Need to Know

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The kidney is affected by many chemicals. Some of the chemicals may even contribute to end-stage renal disease and thus contribute considerably to health care costs. Because of the large functional reserve of the kidney, which masks signs of dysfunction, early diagnosis of renal disease is often difficult. Although numerous studies aimed at understanding the mechanisms underlying chemicals and drugs that target various renal cell types have delivered enough understanding for a reasonable risk assessment, there is still an urgent need to better understand the mechanisms leading to renal cell injury and organ dysfunction. The increasing use of *in vitro* techniques using isolated renal cells, nephron fragments, or cell cultures derived from specific renal cell types has improved our insight into the molecular mechanisms involved in nephrotoxicity. A short overview is given on the various *in vitro* systems currently used to clarify mechanistic aspects leading to sublethal or lethal injury of the functionally most important nephron epithelial cells derived from various species. Whereas freshly isolated cells and nephron fragments appear to represent a sufficient basis to study acute effects (hours) of nephrotoxins, e.g., on cell metabolism, primary cultures of these cells are more appropriate to study long-term effects. In contrast to isolated cells and fragments, however, primary cultures tend to first lose several of their *in vivo* metabolic properties during culture, and second to have only a limited life span (days to weeks). Moreover, establishing such primary cultures is a time-consuming and laborious procedure. For that reason many studies have been carried out on renal cell lines, which are easy to cultivate in large quantities and which have an unlimited life span. Unfortunately, none of the lines display a state of differentiation comparable to that of freshly isolated cells or their primary cultures. Most often they lack expression of key functions (e.g., gluconeogenesis or organic anion transport) of their *in vivo* correspondents. Therefore, the use of cell lines for assessment of nephrotoxic mechanisms will be limited to those functions the lines express. Upcoming molecular biology approaches such as the transduction of immortalizing genes into primary cultures and the utilization of cells from transgenic animals may in the near future result in the availability of highly differentiated renal cells with markedly extended life spans and near *in vivo* characteristics that may facilitate the use of renal cell culture for routine screening of nephrotoxins. — *Environ Health Perspect* 106(Suppl 2):559–569 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/559-569pfaller/abstract.html>

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Introduction

Exposure to drugs and chemicals often results in toxicity to living organisms. We must recognize the fact that not all compounds are equally toxic to all parts of a living system because the toxic actions of many compounds are manifested in specific organs. These organs are known as target organs of toxicity. This concept has developed into the evaluation of toxicants via their target organ specificity.

Several factors determine the susceptibility of a particular organ to toxicity. These factors include the pharmacokinetics of the compound, the metabolic fate of the compound, and the target organ's ability to respond to the toxic insult. Many organs, including the kidney, are capable of metabolizing chemicals to toxic reactive intermediates. The *in situ* metabolic activation of chemicals results in selective toxicity. These

reactive intermediates can initiate toxicity via binding to cellular macromolecules or via generation of reactive oxygen species, which may lead to peroxidative damage of functionally crucial cellular structures such as membranes or nucleic acids.

The kidneys are dynamic organs and represent the major control system maintaining body homeostasis, i.e., water and electrolyte balance. Although they comprise less than 1% of total body mass, they receive about 20% of the resting cardiac output. The nature of renal structure and function renders the kidneys especially susceptible to toxic xenobiotics. The high rate of blood perfusion and the well-developed transport systems for ions and solutes, as well as the capability to recover water and thereby concentrate the solutes to be excreted from the body, make the kidneys extremely vulnerable to xenobiotic-induced toxicity. This is perhaps best illustrated by the fact that end-stage renal disease (ESRD) represents a major burden to European health care systems. The health care costs for diagnosed ESRD patients in Europe amount to more than 3500 million European currency units per year. Approximately 30% of ESRD results from infectious or genetic diseases and approximately 20% result from therapeutic agents (1). The majority (50%) of the diagnosed cases of ESRD, however, are of unknown etiology (Table 1). Current consensus suggests that chemicals and drugs probably play a significant role in those degenerative conditions for which there currently is no documented etiology.

It is difficult to establish the etiology of these cases of ESRD because humans are exposed to many potentially nephrotoxic chemicals, any one of which may take up to several decades to produce clinical symptoms. This is especially so with renal disease, as it is difficult to diagnose at an early stage when causality could be more clearly understood.

Table 1. Causes and costs of end-stage renal disease in Europe.

Percentage	Cause	Costs to Europe ^a
20%	Therapeutic agents and chemicals	711
30%	Disease, microorganism, genetic disorders	1067
50%	Unknown etiology	1778
Total	All causes	3556

^aIn million European currency units.

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Abbreviations used: ATP, adenosine triphosphate; ESRD, end-stage renal disease; GSH, glutathione; IPK, isolated perfused kidney; LLC-PK₁, porcine cell line; MDCK, Madin–Darby canine kidney; OK, opossum kidney.

Role of Nephron Heterogeneity in Nephrotoxicity

A number of normal biochemical and physiologic patterns can be identified that make the cells of the kidney, compared with those of other organs, especially susceptible to ischemic or toxic insults. The major activity of the epithelial cells of the nephrons (uriniferous tubules), the kidney's functional units, is reabsorption of Na^+ from the blood plasma filtrate produced in the renal glomeruli. This process represents the driving force for reabsorption of water and the coupled uptake of organic solutes such as sugars and amino acids. Reabsorption of sodium against an electrochemical gradient

is active and mediated by the basolaterally localized Na,K-ATPase , a pump fueled by cellular adenosine triphosphate (ATP) derived predominantly from oxidative metabolism of the epithelial cells (2). The linear correlation between renal oxygen consumption and Na^+ reabsorption is generally assumed to result from direct coupling between energy providing metabolism and energy consuming transport processes and illustrated by the morphologic layout of renal epithelial cells along the various nephron segments (3) (Figure 1).

The major source of renal epithelial cell energy is oxidative breakdown of metabolic substrates. However, in addition to aerobic energy production, other metabolic pathways such as glycolysis deliver significant

quantities of metabolic energy for renal function (4,5). Enzymes of the glycolytic pathway are confined to the distal nephron portions, which makes cells there relatively resistant to oxygen deficiency as compared to proximal tubular cells. The capability to *de novo* generate glucose is restricted to the proximal nephron (6), which can also oxidize α -ketoglutarate, glutamine, glutamate, malate, and succinate, but not glucose and lactate, to CO_2 and water. In contrast, cells of the medullary thick ascending limb can oxidize glucose and lactate as well as glutamate, α -ketoglutarate, and palmitate. However, they are unable to utilize succinate or malate (7). Because substrate oxidation along the nephron is dependent on the distribution of the respective enzymes (8), distal nephron portions predominantly will use glucose for maintenance of their cellular ATP whereas proximal nephron portions preferentially use glutamine. The mammalian kidney further shows a remarkable substrate specificity and selectively extracts these substrates from the blood supply in considerable amounts. Long and short chain fatty acids, as well as citrate and ketone bodies, complete the substrates listed above as major energy sources for renal work (4).

The anatomical, functional, and biochemical differences among the various cell types along the nephron imply that these cells will each respond in a characteristic manner to exposure to chemical toxicants or to pathologic conditions such as hypoxia or ischemia. Indeed, the kidneys are prime targets of several drugs, toxic xenobiotics, or chemicals; first, because of the high blood flow rate and second, because of the presence of cellular transport systems that facilitate concentration of these compounds within the nephron epithelial cells. Because of the heterogeneous distribution of transport systems and different abilities to either bioactivate or detoxify xenobiotics, the different cell populations (and therefore the various regions within the kidney) will not all display the same degree of damage after exposure. Rather, distinct cell populations may be particularly susceptible to a specific class of chemicals and resistant to injury from another class of chemicals. Renal epithelial cells of the proximal nephron are target sites for many nephrotoxic chemicals because of a large number of transport systems (8) and because of the presence of xenobiotic metabolizing enzymes such as cytochrome P450, reduced nicotinamide adenine dinucleotide phosphate-cytochrome C reductase, glucuronyl transferase, sulfotransferases,

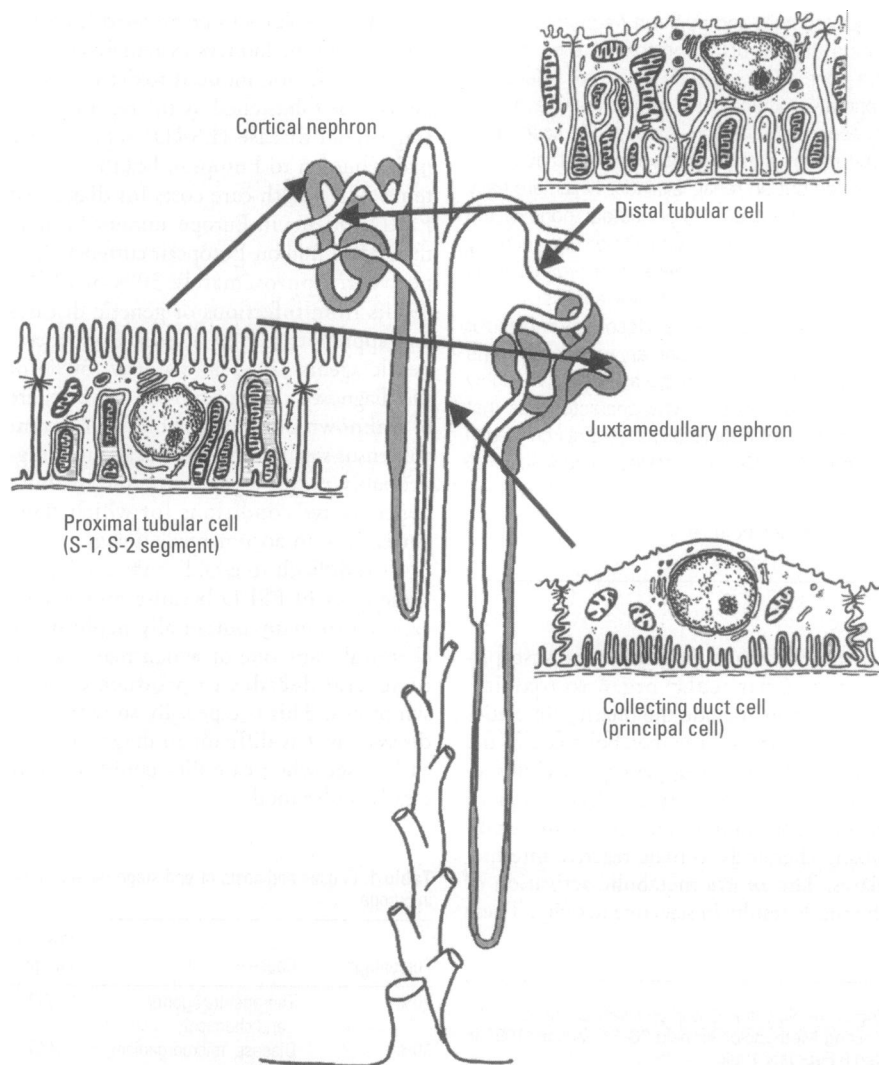


Figure 1. Schematic of two nephrons (cortical and juxtamedullary). The cell types shown are restricted to those most widely used for either isolation or establishment of primary cultures: a proximal tubular cell from the convoluted portion, a cell from the distal convoluted tubule, and a principal cell from the cortical collecting duct.

glutathione S-transferases (including cysteine conjugate β -lyase), monooxygenases, and prostaglandin H synthase (8-11). In addition, intracellular concentrations of reduced glutathione (GSH) and GSH-dependent enzymes are highest in this nephron segment (8). Therefore, injuries that alter cellular redox state, such as oxidative stress during ischemia-reperfusion injury, will vary along the nephron. Variations are also expected according to the differences in cellular energy metabolism, which displays a similar distribution pattern to that outlined for GSH. Indeed, early proximal nephron segments (S-2 segments) appear to be more resistant to oxidative injury than their outer medullary portions (S-3 segments) and the thick ascending limb of Henle's loop (8).

The cellular and molecular mechanisms of a limited number of nephrotoxic lesions are now sufficiently well understood to allow rational risk assessment to be undertaken. A list of the major classes of nephrotoxins known to affect animals and humans follows:

Therapeutic agents

- Analgesics
 - Nonsteroidal antiinflammatory drugs
 - Paracetamol
- Antibiotics
 - Aminoglycosides
 - Cephalosporins
 - Amphotericin B
 - Tetracyclines
 - Puromycin
- Lithium
- Urographic contrast media
- Anticancer drugs
 - Cisplatin
 - Adriamycin
 - Ifosfamide
- Immunosuppressive agents
 - Cyclosporin A
- Heroin

Chemicals

- Ethylene glycol
- Organic solvents
 - Volatile hydrocarbons
 - Chloroform
 - Light hydrocarbons
- Bipyridylic herbicides
- Mycotoxins
 - Ochratoxin-A
 - Citrinin
 - Orellanine
- Silicone
- Metals
 - Arsenic
 - Bismuth
 - Cadmium
 - Chromium

- Germanium
- Gold
- Lead
- Mercury
- Uranium

Following chemical injury, tissue responds dynamically and, where possible, repairs occur rapidly. The repair potential decreases as the changes in renal cellular function move from biochemical perturbation to primary and secondary injury (Figure 2). At the same time the capability to compensate injury decreases. If the changes are rapid, acute renal insufficiency or failure is the result. If the injurious processes develop slowly, chronic degenerative changes occur and the capability to compensate for functional changes—the organ's functional reserve—declines steadily.

The kidney's role in regulating body homeostasis may mask functional changes that are transient and hence go unnoticed. Because of functional and morphologic heterogeneity, the cellular response to injury differs depending on the region of the kidney affected. Although the majority of the epithelial cells lining the uriniferous tubules repair rapidly, the cells belonging to the renal glomerulus, which is responsible for ultrafiltration of blood plasma and production of primary urine, and special cells contained in the renal medulla, involved in concentration of the primary urine, are less capable or are unable to repair. Repeated insults to regions that do not repair or show only slow repair will progressively erode those cells over years and lead to chronic renal failure and ESRD. Nephrons undergoing insults have

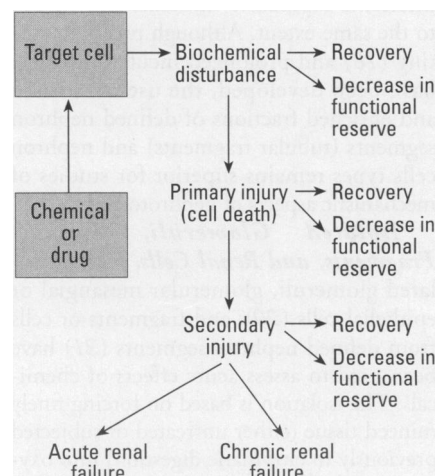


Figure 2. Scheme of the target-selective nature of renal injury and the subsequent cascade of degenerative changes.

a substantial capacity for compensating injury—renal functional reserve. This makes the assessment of the degree of functional impairment difficult. Standard function tests like the determination of the glomerular filtration rate or the reabsorption of water and electrolytes are indicative of extensive damage and measure change only when the functional reserve capacity has been eroded beyond a critical value. Substances to be eliminated by renal excretion, such as urea or creatinine, will increase their blood level only if 70 to 80% of the renal epithelial mass has been lost (Figure 3). At present no procedure for assessment of renal functional reserve is available. The destruction of one renal epithelial cell type could (if compensation for lost function is not possible) compromise the adequate function of other cells and thus trigger a cascade of degenerative changes in adjacent or even more distant cells. As long as cell damage is below a certain threshold or is sublethal, functional disturbance may become measurable only if sensitive enough methodologies are applied. These signs of injury are the release of enzymes or other protein molecules from damaged renal cells into the urine, which potentially offer the noninvasive diagnosis of renal damage (12). However, these procedures are to the best of our knowledge only useful for detecting acute events. Such injuries are usually either repaired or compensated.

Only if the duration of insults is sustained and damage to particular renal cell types is not repaired will renal failure develop over a prolonged period. Under these conditions slow progressive changes occur and the kidney is unable to function fully. This is most often recognized by the inability to eliminate end products of metabolism such as urea or creatinine from the body fluids via urinary excretion. Again, these changes are apparent at an advanced stage of renal dysfunction.

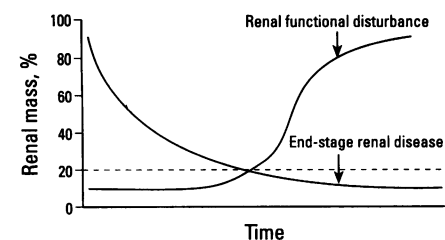


Figure 3. Schematic of the staging of renal failure.

Monitoring of low- and high-molecular-weight proteins in the urine can identify injury of the filtration units, the glomeruli, or the uriniferous tubule epithelium relatively early during development of chronic renal failure.

In Vitro Screening and Mechanistic Studies

Caused by the target organ-selective nature of many nephrotoxic xenobiotics, *in vitro* methods have become an invaluable tool for understanding mechanisms of action. However, there is need to carefully match the *in vitro* system and the conditions to the question being asked. In this context there are problems not yet readily solved, e.g., the most relevant concentration of a chemical. Of importance is that the intact kidney possesses the capability to concentrate several compounds during urine formation to a degree that may exceed plasma concentration by a factor of 10^2 to 10^3 , and that this process cannot be assessed analytically for every potentially toxic compound.

A number of end points, e.g., enzyme leakage from cells; alterations in solute transport across cultured renal epithelial cell layers; their energy metabolism; the synthesis, expression, and turnover of a number of specific cellular marker molecules; and light and ultrastructural pathology, have been applied to the *in vitro* techniques outlined in more detail below.

Higher Order Systems

The Isolated Perfused Kidney. The isolated perfused kidney (IPK); usually taken from rat or rabbit), either perfused cell free or in the presence of erythrocytes (13), is the most appropriate system for studying potentially nephrotoxic xenobiotics (14) when tubulovascular integrity is required. This system is not influenced by higher order regulatory systems (e.g., nervous, hormonal, and blood-borne factors). The IPK enables precise control of the concentration of compound that is being studied. However, it is not a system to be used for routine examinations. The disadvantage, compared to studies of the kidney *in situ*, is that renal function is maintained for only a short period of time (approximately 2 hr), although the life span can be prolonged considerably by the use of sterile tubing or the addition of oxyphoretic compounds or erythrocytes (13). If tissue oxygenation is provided only by physically dissolved oxygen (i.e., blood-free perfusion), the IPK already represents a hypoxic model of renal function and is further

characterized by perfusate flow rates far above those *in vivo* and *in situ*. Filtration rate, sodium, solute, and water reabsorption, on the other hand, are below the usual *in vivo* values. Three modes of perfusion can be utilized: single path perfusion, recirculation of the perfusate, or recirculation and dialysis of the perfusate. Dialysis often is combined with oxygenation (15).

Isolated Perfused Nephrons. Isolated perfused nephrons (16), or more precisely nephron segments, have delivered valuable insights to functional (transport, electrical properties) characteristics of certain nephron segments. However, this technique is not really suitable for routine *in vitro* nephrotoxicity studies. Investigation of microdissected nonperfused segments has provided knowledge on localization of enzyme systems (8), metabolic pathways, and the distribution of receptors that are now being used to confirm the site of origin of renal cellular systems after isolation.

Renal Tissue Slices. One of the earliest *in vitro* techniques applied and still widely used for the study of transport and toxicity is the renal tissue slice (17). A substantial portion of our current knowledge on nephrotoxic mechanisms has been derived from this technique. However, renal slices present several disadvantages. First, slices contain a heterogeneous cell population, which makes assessment of functional changes upon exposure to a toxicant in a specific cell type quite cumbersome. Second, many of the cells and the exposed surfaces are damaged from cutting. Third, slices still represent a complex conglomerate of various cell types interacting with each other and do not ensure that every cell of interest is exposed to nutrients and oxygen to the same extent. Although precision cutting (18) and prolonged incubations (19) have been developed, the use of isolated and enriched fractions of defined nephron segments (tubular fragments) and nephron cells types remains superior for studies of mechanistic aspects of nephrotoxicity.

Isolated Glomeruli, Tubular Fragments, and Renal Cells. Freshly isolated glomeruli, glomerular mesangial or epithelial cells (20), and fragments or cells from defined nephron segments (21) have been used to assess acute effects of chemicals. The isolation is based on forcing finely minced tissue (either untreated or subjected previously to enzymatic digestion) into oxygenated buffer through a series of sieves (22). The purity of the glomerular and tubular preparation is high and can be further improved by subjecting the fragments

to percoll isopycnic centrifugation (21). Alternatively, preparation yield can be improved by infusion of iron particles prior to the separation of tubular fragments to completely remove the vascular components and glomeruli by magnetic separation (23). The glomerular and tubular preparations obtained can then be submitted to a number of unspecific but sensitive end points of toxicity testing as well as to a number of test procedures specific for the respective cell type of interest.

For glomeruli, sensitive end points would include metabolism and the synthesis of proteoglycan, collagen, and fibronectin; with proximal and distal nephron fragments or isolated cells, studies of the effect of nephrotoxins on metabolism and transport studies can be undertaken. Such studies have been performed with fragments and cells isolated from rat, rabbit, pig, dog, and human kidneys. The advantage of using fragments is that the *in vivo* architecture of the epithelium is retained. Therefore, alterations in nephron segment-specific biochemical functions after injury induced by toxic xenobiotics can be easily obtained, leading to a better understanding of nephrotoxic mechanisms. Using this approach numerous studies on mechanistic aspects of nephrotoxicity have been conducted, e.g., cephalosporins, angiotensin-converting enzyme inhibitors (24), or cisplatin (25), to give just a few examples. Similarly, isolated cells of different nephron origin have been used for that purpose (26).

A limitation of this method is that the fragment ends represent damaged sites, thus the life span is limited to a few hours. Isolated nephron cells suffer from the same disadvantage. For longer lasting studies, primary cultures of cells are needed.

Cell Culture Models in Nephrotoxicity Testing

Cell culture techniques as a tool for *in vitro* nephrotoxicity studies have gained more importance over the past two decades. This is largely due to improved methodologies for growing homogeneous cultures of renal cells. Two major strategies have been pursued: the use of primary cultures of glomerular mesangial and epithelial cells and renal epithelial cells from various sites along the nephron, and the use of permanent renal epithelial cell lines.

Depending on the retention of adequate renal cellular functions, which interfere with xenobiotic or drug action (27), renal cell cultures have the advantage of providing an experimental model uninfluenced by

higher order regulatory systems. For successful applications of cell and tissue cultures in renal physiologic and toxicologic studies, the following requirements must be met:

- Cells should retain the polar architecture and junctional assembly of epithelia and should express the proper polar distribution of membrane enzymes and transport systems (28), resulting in the exhibition of vectorial transport of solutes and water manifested by the formation of domes (29) and the generation of transepithelial electrophysiologic characteristics (such as a spontaneous transepithelial potential difference) resulting from a certain transepithelial resistance (Figure 4) and short circuit current (30).
- The cellular uptake of xenobiotics should occur from either the apical or basolateral side as observed *in vivo* (31).
- Cultured cells should retain nephron segment-specific characteristics, distinct metabolic and transport properties, and hormone responsiveness (32).

These requirements are best, though not completely, met by primary cultures of renal epithelial cells during the early period after initiation of cultures. None of the continuous renal epithelial cell lines used, however, fully express all the needed differentiated functions known from the ancestor cell(s) *in vivo* and *in situ* (32).

Obtaining primary cultures from the kidney is somewhat hampered by the fact that there are at least 15 to 20 cell types that comprise the kidney and the nephron, respectively (33). One must ensure that homogeneous cultures have been obtained before any study can begin (34).

Despite this difficulty, primary cultures of proximal nephron epithelium from various species have been developed, i.e., mouse (35), rat (36,37), rabbit (38), and

pig (39). Although cells in primary culture tend to dedifferentiate within hours, the characteristics of those cells are usually closer to the *in vivo* situation as compared to cell lines.

The methods of isolation used are bulk isolation of nephron fragments or of cells by the methods described above (mechanical disruption, sieving, digestion, and isopycnic centrifugation). The enriched tubular segments or cell types must then be submitted to appropriate media to either maintain proliferation or to select for specific cell types.

Precise knowledge of the segmental metabolic (4,40) and hormonal (41,42) properties along the mammalian nephron allows application of specific metabolic tissue culture conditions to select the nephron cell type of interest. D-Amino acid (D-valine)-containing culture media can be used to select for cells that express D-amino acid oxidase activity and prevent the culture overgrowth by fibroblasts (43). Glucose-free pyruvate-supplemented culture media will provide growth only for gluconeogenesis-competent proximal tubular cells (44-46). For the isolation of papillary collecting duct cells, their ability to resist a hypoosmotic shock was used to remove noncollecting tubule cells from a papillary digest (47).

The most sophisticated metabolic and endocrinologic approach developed to specifically select certain cell types is the design of serum-free hormone-supplemented culture media (48). Today, hormonally defined media descriptions are available for culturing cells of almost all nephron segments of the most widely used mammalian species (34,36,49-51) including human (52).

Bulk isolation can be combined with immunodissection and immunoselection, an innovative protocol in the setup of renal primary cultures. This method uses specific antibodies as immunoaffinity reagents to isolate populations of different renal cell types. As the number of polyclonal and/or monoclonal antibodies directed against cell surface antigens unique to different renal cell types steadily increases [a set of cell-specific monoclonal antibodies has already been described (53-55)], this immunologic procedure will become a widespread tool in establishing primary cultures of defined subpopulations of cells, especially from distal and cortical collecting tubule portions (56,57). The most advanced methodology to establish ultrapure primary cultures of proximal and distal tubular cell populations was recently described by

Helbert (58), who used immunodissection in combination with flow cytometry to establish renal primary cultures of proximal tubular S-1, S-2, and S-3 segment cells from the human kidney.

A more laborious method is the visual selection of individual tubular segments (16) and outgrowth under appropriate culture conditions (34).

One of the disadvantages of establishing renal primary cultures is that there is still a considerable gap in available information with respect to markers that can be used to unambiguously identify the respective *in situ* ancestor cell (32), in particular those of glomerular origin, after bulk isolation of cells by centrifugation. Furthermore, the situation is complicated by the fact that cells tend to quickly dedifferentiate as they are maintained in culture.

Furthermore, not all nephron cells isolated so far can be passaged during primary culture either because of dedifferentiation or because of the unavailability of adequate culture media. For example, growth of primary cultures of rat proximal tubules is extremely difficult (59). Some improvement has been achieved by utilizing microporous growth supports (60) that ensure nutrient access from the apical as well as the basolateral side of cultured monolayers. In addition, coating the growth substrates with extracellular matrix molecules such as collagen I, collagen IV, fibronectin, laminin, or commercially available mixtures of these compounds (e.g., Matrigel, Becton Dickinson Labware, Bedford, MA) (61) appears to improve adhesion, proliferation, and differentiation of cells in culture. Despite the limitations outlined, renal primary cultures represent a reliable though not easy to handle strategy to study basic renal cellular functions and their modulation by nephrotoxicants (62).

Primary cultures have been successfully used to study *in vitro* effects of cisplatin, gentamycin, cephalosporins, cysteine conjugates, butylhydroperoxide, mercuric chloride, and cadmium chloride. (37,63-66). The majority of *in vitro* nephrotoxicity studies, however, have been performed on permanent or continuous renal epithelial cell lines, which have been powerful tools in the study of nephrotoxicity *in vitro*. The most widely used lines and their sites of origin are discussed below (Table 2). In addition to many advantages, such as the possibility of long-term exposure under controlled conditions, the unlimited life span, and the lack of time-consuming isolation procedures, cell lines suffer from

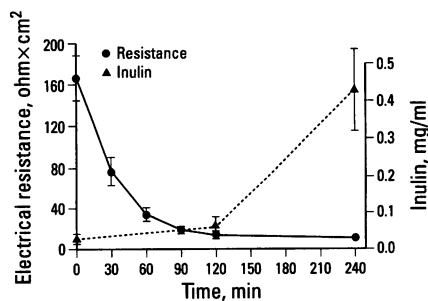


Figure 4. Effects of 200 μM CdCl_2 on the transepithelial resistance and paracellular permeability of LLC-PK₁ monolayers grown on a microporous support.

Table 2. Continuous renal epithelial cell lines.

Cell line	Animal derived from	Nephron segment of origin
LLC-PK ₁	Yorkshire pig	Proximal nephron
OK	North American opossum	Proximal nephron
JTC-12	Monkey	Proximal nephron
MDCK	Dog (cocker spaniel)	Collecting duct
A6	<i>Xenopus laevis</i>	Distal tubule/collecting duct

Abbreviations: LLC-PK₁, porcine cell line; MDCK, Madin-Darby canine kidney; OK, opossum cell line.

some drawbacks. Although they have retained a number of differentiated functions from their *in vivo* ancestor cells (32), they have dedifferentiated in culture. As a result, cells claimed to be of proximal tubular origin exhibit a combination of properties characteristic of different parts of the nephron. Two cell lines that are often used as models for the proximal nephron are the porcine cell line (LLC-PK₁) and the opossum kidney (OK) cell line. The LLC-PK₁ cells lack expression of the enzyme fructose-1,6-bisphosphatase, rendering them incapable of gluconeogenesis, which is a key metabolic pathway in proximal nephron cells (38). In addition, LLC-PK₁ cells are not responsive to parathyroid hormone and lack a probenecid-sensitive organic anion transporter (67). OK cells, on the other hand, display little γ -glutamyltranspeptidase and lack alkaline phosphatase; both enzymes are considered markers for the proximal nephron (32). To improve this situation our laboratory succeeded in developing gluconeogenic strains of both cell lines by adaptation of cells to glucose-free media (45,46). It may be speculated from these results that by using appropriate culture conditions, including the well-defined extracellular matrix, continuous cell lines could be redifferentiated to some extent. Cellular and molecular biology technologies such as cell fusion or transfection techniques could be used to reexpress lost functions, e.g., specific transporters or enzymes or perhaps even specific receptors.

A combination of the advantages of a continuous cell line with the better differentiation of primary cultures can be obtained by immortalization of the latter. Immortalization of primary proximal tubular cell cultures has been achieved in several ways (68). Proximal nephron cell lines have been produced by targeted oncogenesis in transgenic mice using a pyruvate kinase-SV40(T) antigen hybrid gene (69). Transduction of the SV40 large T antigen has also been used to establish cell lines from rat primary proximal nephron cell cultures of the Wistar Kyoto rat (70) and the rabbit (71,72). Immortal human proximal

tubular cell lines have further been generated by transduction with human papilloma virus (HPV 16) *E6/E7* genes (73). Using a hybrid adeno 12-SV40 vector (74), successful immortalization of rabbit (75) and human proximal primary cultures was achieved (76). It remains to be established whether these newly developed lines have maintained all the desired characteristics of their *in vivo* precursor cells and are able to retain their functional characteristics through multiple passages.

Culture Conditions for *in Vitro* Nephrotoxicity Testing

Culture media composition, including serum, hormones, growth factors, culture substrata, and extracellular matrix components, substantially influence the expression of specific morphologic features and functions and thus the degree of cell differentiation. The expression of membrane enzymes and transport systems varies significantly with culture duration (77). It is therefore essential to precisely define the culture conditions for each cell line used and for each functional parameter studied in physiologic or toxicologic experiments.

Another major factor influencing cell shape, size, and degree of differentiation is the culture substratum, either the extracellular matrix (78) or the physical tissue culture support (79). The degree of morphologic and functional differentiation of epithelial cells from different tissues increases considerably when the epithelia are cultured on permeable surfaces, thereby providing free access of tissue culture medium to the basolateral membrane surface (79). This technique of culturing transporting epithelia represents a prerequisite for studying epithelial dysfunction for *in vitro* nephrotoxicity testing (80).

Physiologic and Biochemical Functions in the Assessment of *in Vitro* Nephrotoxicity

A major route through which xenobiotic compounds are processed for excretion in the kidney is conjugation with GSH

resulting in the formation of mercapturic acids (81). This process is associated with the formation of highly reactive nephrotoxic compounds (82). In particular, GSH and cysteine *S*-conjugates have been identified as nephrotoxins. In several studies, cysteine *S*-conjugate transport and toxicity have been elaborated *in vitro* on LLC-PK₁ cells derived from the pig proximal nephron (83) and rat proximal primary cultures (84). LLC-PK₁ cells respond to toxic GSH and cysteine *S*-conjugates and bio-transformed cysteine *S*-conjugates respond to toxic mercapturates. Furthermore, these conjugates are taken up by a basolateral amino acid transport system (83). Recently, mercapturic acid formation was also shown in OK cells (85). Thus, cultured epithelial cells may serve as model systems to study aspects of renal biotransformation (86) and the resulting nephrotoxicity and nephrocarcinogenicity (87). Nephrotoxic and mutagenic cysteine conjugates are activated by the enzyme cysteine conjugate β -lyase to reactive acylating compounds that covalently bind to cellular macromolecules. In LLC-PK₁ cells, *S*-(1,2-dichlorovinyl)-L-cysteine induced mRNA for *c-fos* and *c-myc*, which could be blocked in part by reducing agents. Buffering of the elevated intracellular Ca²⁺ induced by the cysteine *S*-conjugate could reduce *c-myc* but not *c-fos* induction (88). Thus toxicity resulting from bioactivated xenobiotics appears to selectively trigger intracellular signaling pathways that have pronounced effects on gene expression, e.g., the DNA damage-inducible gene *gadd 153* (89). Depletion of cellular glutathione by reactive electrophiles can activate transcription of the hsp 70 gene family (90). Moreover, nephrotoxic xenobiotics at sublethal concentrations seem to alter expression and release of growth factors such as fibroblast growth factor type-1 and epidermal growth factor (91), which may thereby trigger accelerated repair of renal epithelium (92). Transforming growth factor beta-1, on the other hand, seems to suppress repair (spreading and proliferation) of mercury and dichlorovinyl-L-cysteine triggered damage in rabbit primary proximal tubular cultures (66).

The recent observation of the expression of the multidrug transporter P-glycoprotein (93) in renal epithelial cell lines (94), normally present in the apical membrane of the proximal tubule (95), opens new ways to study multidrug resistance of renal cells and the potential renal elimination of chemotherapeutic agents.

End Points of *in Vitro* Toxicity Testing

Cultured renal epithelia have been successfully used as model systems to study the cytotoxic action of nephrotoxic compounds (32).

Maintenance of monolayer integrity following the administration of a toxic xenobiotic can be monitored easily by using light microscopy analogous to histopathology. Cell integrity can further be determined by measuring the release of marker enzymes of the various cellular compartments into the culture medium, as in urine analysis. The enzymes determined are usually lactate dehydrogenase (80), *N*-acetylglucosaminidase, glutamate dehydrogenase, γ -glutamyltranspeptidase, or alkaline phosphatase (81). One should keep in mind, however, that the toxic compound studied may also inhibit the activity of the marker enzyme (96).

Other parameters that can be used to ascertain the nephrotoxic action of xenobiotics are those indicative of the integrity of transport functions of the cultured epithelium. These parameters include the microscopic observation of domes (97), measurement of epithelial leakage of indicator compounds (98), and monitoring of transepithelial electrophysiologic parameters in Ussing-type chambers (99). These parameters are the spontaneous transepithelial potential difference, the transepithelial resistance, the ion transport-generated short circuit current, and the anion-to-cation permeability ratio as a measure of the shunt path perm-selectivity of the epithelium. In particular, these parameters seem to provide the most sensitive measures of epithelial permeability and thus of the barrier function of the cultured epithelium (100).

The latter parameter in particular has been successfully applied for leaky epithelia such as the proximal nephron (101). Because of the low transepithelial resistance ($100\text{--}400\ \Omega \times \text{cm}^2$) of most of the cultured epithelia and the mammalian proximal tubule, accurate measurements of the transepithelial resistance are difficult to perform and are highly dependent on the geometry of the Ussing-chamber device and on the geometry and reproducibility of the electrode positioning, respectively (102). Thus, quantification of transport and its alteration by toxic xenobiotics by means of electrophysiologic techniques is hampered by the very low spontaneously generated transepithelial potentials and resistances. Despite these facts the differential effects of reactive oxidants on apical and

basolateral membrane domains and epithelial conductance of Madin–Darby canine kidney (MDCK) monolayers have been studied (103). The effects of phorbol ester tumor promoters on epithelial tight junctions (104) and the disruption of intercellular junctions by cadmium (105) have also been studied on LLC-PK₁ epithelia using this approach.

Furthermore, electrophysiologic parameters obtained at the single cell level can be used to assess the cytotoxic action of xenobiotics (106). Recently, the method of continuously recording the cell membrane potential of cultured cells with intracellular microelectrodes was applied to elucidate the mechanism of heavy metal toxicity in LLC-PK₁ cells (107). Measurements of cell membrane fluidity may provide additional information on epithelial membrane function (108). The Na⁺-dependent uptake of glucose (109) and the transepithelial glucose flux were used as sensitive parameters of apical membrane function of injured cultured renal cells (110).

Other parameters that have been successfully used to elaborate nephrotoxicity in experimental animals, including the measurement of cell metabolism and intracellular ion homeostasis, can also be applied in *in vitro* studies of cultured renal cells.

The key role of intracellular calcium in the pathogenesis of renal cell injury in acute renal failure is well established (111). Measurements of cytosolic calcium concentrations in cultured renal cells (112) permit the study of perturbations of calcium homeostasis under different nephrotoxic conditions (113), although the effects of gentamicin on cytosolic calcium are still controversial (114).

As a metabolic parameter, the level of cellular ATP, the main energy source for renal cellular and transepithelial transport (2), is one of the best candidates to assess the metabolic and thus the functional state of normal and injured renal cells (106). A highly sophisticated but noninvasive method to monitor renal cell metabolism and cellular ATP levels *in vivo* is nuclear magnetic resonance spectroscopy. The *in vitro* application of this technique (115) permits the study of the metabolism of freshly isolated and cultured renal epithelial cells (116).

In addition to all these technical approaches, the morphologic assessment of cell damage at light microscopic and ultrastructural levels will still provide valuable tools in studies of renal cell injury (80). The application of electron microscopy

techniques on cultured renal cells allows comparison of xenobiotic-induced cell damage *in vitro* with the situation found *in vivo*, e.g., the effects of gentamycin (80,114), cadmium (105), and mycotoxins (117) on the ultrastructure of cultured cells.

Future Outlook

New promising techniques are currently under development that may improve the applicability of renal epithelial primary cultures. Cells grown on microporous supports under continuous perfusion of culture media appear to better maintain the differentiated state over prolonged periods instead of providing good proliferative properties (118). Furthermore, continuous medium perfusion may lead to the reexpression of lost functions in continuous cell lines. This would permit the culture of freshly isolated human and animal cells under nearly organotypic conditions. It would further allow the use of such cultures for subchronic nephrotoxicity testing and still enable assessment of all possible toxicity end points. Coculturing renal epithelial cells with either endothelial or immune cells under perfusion conditions would allow testing of important signal molecules (119) (released from coculture partner cells such as cytokines) that are used in therapy (interferons, interleukins, immune cell growth factors) and produced by the biotechnology industry. Because of the high species specificity of these latter compounds, *in vitro* test systems based on human cells (primary or continuous) will become mandatory to judge toxic side effects and therapeutic risks, as testing on animal models will be impossible.

Nephrotoxic side effects of xenobiotics can be tested by cell culture techniques. The two most important applications are screening of newly synthesized drugs or environmental contaminants suspected of exerting adverse effects on the kidney, and investigation of the mechanistic aspects leading to renal cell injury. Especially with respect to the latter, renal epithelial cultures offer the possibility of easy access to the object of interest, the cell lines are provided in nearly unlimited amounts, and they match their site of nephron origin reasonably well. In this context, continuous renal cell lines represent the experimental system of choice at present. They are easy to grow, maintain, and handle, are commercially available (e.g., from the American Type Culture Collection, Rockville, MD), and retain most of the basic functions of their ancestor cell, at

least in case of permanent proximal and collecting duct cells (LLC-PK₁, OK, JTC-12, MDCK, A6). Another advantage is that an enormous amount of information about culture conditions and differentiated functions (e.g., metabolism, transport, and hormone responsiveness) is available from the literature (32). A disadvantage is the fact that cell lines may suffer from loss of one or the other *in vivo* and *in situ* functions as a result of prolonged cultivation. Under these circumstances, or if one of the lost functions seems to be the predominant target for a nephrotoxic xenobiotic under investigation, the more laborious and difficult application of primary cultures must be done. In addition, one must keep in mind that continuous cell lines are heterogeneous, and multiple cell types might be present in an uncloned wild-type culture.

Therefore, methods should be developed to reexpress the lost functions or to tailor new cell lines more closely matching the cell type of origin in continuous cell lines. Such an enterprise may include several cell biology techniques already available.

The most simple approach would be adaptation to culture conditions that more closely resemble the *in vivo* environment of the respective cell type. As mentioned, omission or drastic reduction of glucose and replacement against pyruvate in the media used for cultivation of LLC-PK₁ cells enables reexpression of gluconeogenesis (45,46). Fusion of cells of continuous cell lines (120) stemming from the same *in vivo* ancestor cell of different species and with different retention of cellular functions, i.e., metabolic pathways, transport systems, or hormone receptors, may be used to establish new

continuous lines more closely resembling the characteristics of the cell type of origin. Fusion of cells from primary cultures with cells from continuous lines delineated from the same nephron cell or nephron segment may help to either immortalize the respective primary culture or help to reexpress lost functions within the continuous cell type.

Genetic approaches (68,121) may deliver another route to establish new, more natural permanent cell lines. Transfection with different but defined genes will be one of the important tools (68,122).

These strategies should even offer the possibility of establishing cell lines expressing most or all of the functions of human renal epithelia. However, all of these trials demand careful selection procedures to isolate the fused hybridoma, the mutated, or the transfected cells.

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