Morphogenesis of Epithelial Neoplasms Induced in the Rat Kidney by Dimethylnitrosamine

G. C. Hard and W. H. Butler

INTRODUCTION

DMN\(^2\) induces 2 types of neoplasms in the rat kidney (25). One is a mesenchymal tumor originating in periglomerular fibroblastic cells of the interstitial space (14–16), while the 2nd is an adenocarcinoma, identifying in many cases with the proximal convoluted tubules (14, 18). Mesenchymal tumors, whether induced by DMN or cycad derivatives, are predominantly associated with the immature rat, while adenocarcinomas, although of lower incidence following treatment of the newborn, are found with equal frequency in the immature and mature rat (19, 21, 28, 34).

Conditioning rats with a protein-free diet before treatment with DMN, which reduces the acute lethality of DMN, results in an increased incidence of renal tumors (33). This is due to an increase in the incidence of mesenchymal tumors rather than adenocarcinomas, for with a single injection of DMN, 60 mg/kg, 100% of the surviving, protein-deprived rats developed mesenchymal tumors while 31% simultaneously developed adenocarcinomas (14). At a dosage of 30 mg of DMN per kg in conventionally fed rats, 26% of the survivors developed mesenchymal tumors and 20% developed adenocarcinomas, whereas in protein-deprived rats receiving DMN, 50 mg/kg, the percentages of tumor incidence were 83 and 24%, respectively. Adenocarcinomas and mesenchymal tumors often coexisted in the same kidney with collision tumors.

The high incidence of renal mesenchymal tumors following a single dose of carcinogen provided a very satisfactory model for tracing the sequential development of this complex neoplasm (15, 16), which could be correlated with a morphological analysis of the final tumor. In the acute phase of the injury induced by DMN, changes were also found in the cortical epithelium. It was possible to identify some lesions leading to the development of adenocarcinomas despite their comparatively low incidence, and it is these that we report here.

MATERIALS AND METHODS

The preparation of DMN and rats, the experimental design, and the preparation of material for light and electron microscopy have all been described in detail in preceding papers (14, 15, 17). In brief, male Wistar rats of the Porton strain weighing 90 to 110 g were given i.p. injections of DMN, 30, 50, or 60 mg/kg, after 1 week of being fed a protein-free diet in the case of the 2 higher doses. Rats were prepared for light and electron microscopy first at daily intervals from 24 hr and then at weekly and finally monthly intervals until adenocarcinomas were well developed. Fixation was commenced by intravascular perfusion either with glutaraldehyde or a mixture of glutaraldehyde and osmium tetroxide. Minute areas of tissue relevant to the study were selected by light microscopic examination of whole kidney sections, and the equivalent areas on the Epon block were sectioned for electron microscopy. Apart from routine staining of paraffin-embedded material described in the previous papers, some Epon-embedded tissue was stained with PAS reagent (7).
RESULTS

The kidneys of control rats fed a protein-free diet for 2 weeks showed no alteration in fine structure. In rats treated with 50 and 60 mg of DMN per kg, signs of intracellular damage were evident in the 1st segment of the proximal convoluted tubules as early as 24 hr. Aggregations of large lipid droplets appeared at the bases of these epithelial cells (Fig. 1), while centrally there were foci of cytoplasmic rarefaction and early formation of cytosegresomes (Fig. 2). Some mitochondria were swollen with a loss of cristae and density as the damaged organelles became sequestered within a cytosegresome. Dense lipid-like material was often seen within the altered mitochondria.

By 3 to 4 days, cytoplasmic protrusions into the intertubular space appeared at the bases of damaged epithelial cells (Fig. 3). These were first seen as irregular processes containing vacuoles with dense material or cytosomes and producing a kink in a now abnormally thickened basement membrane. Large convolutions of thickened basement membrane were also seen beneath damaged tubule cells, some containing cytoplasmic protrusions (Fig. 4), while in others the intervening space between basement membrane loop and cell membrane was filled with dense or clear vesicles, granules, and membrane profiles (Fig. 5). Such aggregations appeared to have formed from the pinching off and dissolution of the cytoplasmic protrusions from the basal part of affected proximal tubule cells. These cells also exhibited a loss of apical microvilli and basal interdigitations with the appearance of condensations of microfilaments at the cell base (Figs. 3 and 6).

The above changes were also noted in those distal tubules located in the close vicinity of glomeruli, but the progression was slower than in the S1 proximal convoluted tubules. Similarly, in some animals the lower segments of the proximal tubules became affected but never as severely as the 1st segment. In rats treated with DMN, 30 mg/kg, the lesions consisted mainly of lipid droplet formation and generally involved only the S1 proximal tubule cells.

Three to 4 days marked the commencement of a monocyte infiltration into the cortical intertubular space. At this stage, macrophages became associated with the thickened basement membrane loops enclosing debris-like material beneath damaged tubule cells. Macrophage processes containing phagocytic vacuoles were seen within these loops (Fig. 6). The mononuclear phagocyte infiltration appeared to be predominantly a response to the damaged epithelial cells, although sporadic, mildly degenerate fibroblastic cells were also present in some periglomerular aggregations of macrophages. The latter have been described previously (15, 16).

By 7 days, both the epithelial changes and the macrophage reaction had reached a peak of intensity. The latter was mainly responsible for a diffuse increase in mononuclear cells throughout the cortical intertubular space (Fig. 7). Damaged cells were now frequent in the S1 proximal tubules possessing numerous lipid droplets and aggregations of basal debris-like material within thickened tortuosities of basement membranes. Necrotic cells were sporadically distributed throughout this segment and in periglomerular distal tubules (Fig. 7). The sporadic nature of cell death implied that the vast majority of tubule cells showing the alterations described above recovered. Ultrastructurally, dying cells were identified by uniform marked dilation of mitochondria with consequent vacuole formation, condensation of cytoplasmic ground substance, and contraction of cell outline.

Dividing cells were quite frequently seen, especially at 7 days and until tubule regeneration was complete. These were most commonly seen in tubules located next to glomeruli (Fig. 8). Mitoses were noted throughout the period of study but never as frequently as in the early period. Epithelial damage together with the diffuse interstitial cell reaction had completely resolved by 2 or 3 weeks after treatment.

At 3 weeks, mild enlargement of a few tubule cell nuclei was seen, but by 6 weeks nuclei of very large size and often bizarre shape were frequent throughout the cortex (Fig. 9). Most of these occurred in all 3 segments of the proximal tubule and were no more numerous in S1 than in S2 and S3 tubules. Occasional karyomegaly was also seen in cells of distal tubules. Enlarged nuclei were as frequent at 12 weeks, but by 20 weeks their numbers were decreasing. Tubule cells containing increased, enlarged, and abnormally shaped mitochondria were also occasionally seen from 6 weeks on (Fig. 10). These were sometimes associated with small aggregations of mononuclear cells in the intertubular space.

At 6 weeks, the 1st proliferative tubule was seen, although they were more commonly observed at 12 weeks (Fig. 11). These were always located next to a glomerulus, thus relating their development to the tubules, which earlier showed the most severe damage. In some cases at 3 months, neoplastic lesions were quite well developed and, apart from exhibiting growth by expansion, also showed signs of invasive proliferation beyond the edge of the lesion. The proliferative nature of these tubules was demonstrated by an increase in the number of cells within the epithelial lining leading to either obliteration of the lumen (Fig. 11) or papillary projections into the lumen (Fig. 12). Mitoses were observed within such tubules (Fig. 12, inset). In the lesions examined ultrastructurally at 12 to 20 weeks, brush border formation was present not only at the luminal edges of cells but also between cells which did not adjoin the lumen (Figs. 13 and 14). Some of the cells in these lesions appeared to be hydropic, while others contained numerous mitochondria. The latter were sometimes of abnormal shape and contained few cristae. The Golgi apparatus was very prominent in many cells and hypertrophied (Fig. 14). Granular endoplasmic reticulum was usually fragmentary, but polyribosomes were often very numerous. Many cells were binucleate. Some nuclei lacked a peripheral, clumped chromatin pattern, and prominent nucleoli were frequent. The latter were often abnormal, being hypertrophied and sometimes fragmented. Many cells contained numerous, quite large, cytosome-like bodies. Basal infoldings were absent at the periphery of these tubules, but between cells small finger-like interdigitations were present. The proliferations were surrounded by either normal or very thickened basement membrane and small aggregations of lymphocytes and macrophages. The ultrastructural features of such early lesions, particularly the presence of brush border in

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abnormal situations and abnormal nucleoli, conformed to lobules of invasive adenocarcinoma seen between 6 and 12 months (18). Intermediate stages were identical.

At extended intervals after DMN treatment, other small, tubular proliferations were found. Once again these were invariably located next to glomeruli and consisted mainly of pale, swollen cells (Fig. 15), which stained faintly positive with PAS reagent. Between 6 and 12 months, lesions of the same small size were still present. Some appeared to be derived from distal tubules (Fig. 16). The bulk of cells making up the proliferating aggregations were grossly hydrated. Piled-up hydropic cells were seen in continuation with normal distal tubule cells at 1 end of a sectioned tubule. The bulk of the cytoplasm was filled with dispersed, homogeneous granular material, and the organelles were often confined to the periphery of the cell. The mitochondria, compressed beneath the plasma membrane, thus assumed grotesque shapes. Rough endoplasmic reticulum appeared in short fragments and was often dilated. The Golgi apparatus was usually prominent. In addition, a few vesicles, polyribosomes, lipid droplets, cytosomes, and dense myelin whorls were present beneath the plasma membrane and scattered in islands through the homogeneous granular material occupying the cytoplasm. At the luminal borders, short microvilli reminiscent of those in distal tubules were present, but no brush borders were seen in any location. Binucleate cells were common, and nucleoli were prominent. Basal interdigitations were retained in some cells.

**DISCUSSION**

In the literature to date, there has been little correlation between the production of renal epithelial neoplasms and acute tubular toxicity of a carcinogen. Mage and Barnes (26) have drawn attention to the paradox that, despite a high incidence of renal tumors following DMN treatment, no acute structural damage has been consistently recorded in the kidney apart from congestion. Zak et al. (36) interpreted the very enlarged renal tubule nuclei, seen after some weeks in their study, as the earliest morphological manifestation of DMN intoxication. Matthews and Walpole (27) induced renal adenocarcinoma in rats with 4'-fluoro-4-aminobiphenyl and commented on the absence of severe pathological change preceding tumor induction. Likewise, no acute changes could be identified in the kidney following treatment with methylazoxymethanol (37), despite the fact that repeated injections of this compound induce renal neoplasms (22). Recently, it has been shown that fibroblastic cells in the vicinity of the glomerular hilus show mild intracellular damage as early as 24 hr after treatment with a carcinogenic dose of DMN, and this has been correlated with the development of the mesenchymal tumor (16). In the same way, the present study demonstrates that acute toxic injury does occur in the cortical tubules, which leads to a sporadic cell necrosis. The relationship of the acute injury to development of epithelial tumors is emphasized by the occurrence of early proliferative lesions always in tubules located next to glomeruli, the area that suffers the most severe change in the acute stage of intoxication. Adenocarcinoma development is likely to be related to some degree of acute tubular damage in systems other than DMN treatment. For instance, a high incidence of renal epithelial tumors has been reported following X-irradiation (3, 30), and the same agent also induces acute changes in tubular epithelium of the renal cortex (23, 24). Again, Zollinger (38) has recorded that the induction of renal epithelial tumors in the rat kidney by chronic lead poisoning is always preceded by severe renal damage.

The morphological features of the acute damage produced by DMN in the cortical tubules are nonspecific. Formation of lipid droplets and cytosegresomes, loss of brush border and basal interdigitations, and tortuous thickening of basement membrane associated with aggregations of granular and membranous material have been described in a variety of renal intoxications including poisoning by uranyl nitrate (31), mercuric chloride (13), sodium oxalate (10), ethylene glycol (9), and carbon tetrachloride (32) and also in X-irradiation (24) and autoimmune nephrosis (11). Of these, only X-irradiation has been associated with the induction of renal neoplasia. The nature of the granular and membranous material located within the tortuous basement membrane loops beneath damaged epithelial cells indicates that it may represent debris derived from those cells. The various stages seen suggest that basal protrusions from injured cells fragment to form this material. Tubular protuberances are not seen in the renal cortex of normal rats, although they have been described in the rhesus monkey (35). These aggregations of debris are invariably associated with macrophages which themselves are frequently seen to contain phagocytosed material. The beginning of monocuclear phagocyte infiltration into the interstitial cortical space coincides with the appearance of the foreign material beneath the injured tubule cells. A few days later, the peak of tubular damage coincides with a diffuse increase in intertubular cells throughout the cortex, the predominant constituent of which is the macrophage. By 2 or 3 weeks, the signs of epithelial damage as well as the diffuse interstitial cellular reaction have simultaneously resolved. Thus it appears that the macrophage response in the acute phase of DMN intoxication is associated predominantly with the damage sustained by the tubular epithelium. In addition, a few periglomerular hypercellular aggregations persist beyond this stage and are associated with the presence of occasional abnormal fibroblastic cells. The latter are considered to be precursors of renal mesenchymal tumors (15, 16).

The tubule epithelium responds to the sporadic cellular necrosis by a wave of mitosis, particularly in the 1st segment of the proximal tubule. After resolution of the acute epithelial changes, mitotic incidence reverts back to normal. Nevertheless, foci of cell division must be occurring, although undetected, for from 6 weeks tubules showing proliferative alterations are found. This is earlier than recorded by other workers (2, 20, 25, 36), and, doubtless, fixation by perfusion has facilitated the identification of proliferating tubules in this study. Ultrastructurally, these minute lesions represent the very early stages of epithelial neoplasms, for those examined possessed abnormal, hypertrophied nucleoli and abnormal brush border formation, characteristic features of well-formed adenocarcinomas (18). The variable location of these early

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neoplastic tubules next to glomeruli and the presence of abnormal brush border relate their origin to the 1st segment of proximal tubules, the area most severely affected by DMN. It was not possible to demonstrate continuity between the proliferations of hydropic cells (Fig. 15) with the 2nd segment of proximal tubules. The fact that adenocarcinomas and mesenchymal tumors both arise from a periglomerular site suggests that DMN or a carcinogenic metabolite is concentrated in this area of the kidney.

The nature of small proliferative hydropic tubules persisting at extended intervals after DMN treatment (Fig. 15) is uncertain. Some of these lesions are located in distal tubules, specifically those next to glomeruli, thus relating them again to the area of acute damage. Whether such lesions would very slowly progress to become adenomas with time is purely speculative. In a previous report (18), a clear cell adenocarcinoma was not examined ultrastructurally. The homogeneous particles filling some of these cells ultrastructurally resemble very dispersed glycogen granules, an observation supported by the faint PAS-positive reaction of the lesions. Some authors (1, 20) consider that focal glycogen accumulation precedes the development of certain types of renal adenocarcinomas. Glycogen has been identified as a characteristic of human clear cell adenocarcinoma and is considered to be largely responsible for the translucency of these tumor cells (12, 29). However, the small foci of proliferative epithelium related to the 2nd segment found in our study at relatively early stages, possessing the ultrastructure of neoplastic lesions, were not associated with a PAS-positive staining reaction or with the electron microscopic identification of glycogen. However, clear cell carcinomas do occur in the DMN-treated rat kidney, and it is possible that the small PAS-positive hydropic lesions seen at extended intervals after treatment may represent very slowly proliferating examples of this tumor variety. On the other hand, the hydrated nature of many of the constituent cells suggests that they are, in part, degenerate lesions.

The random distribution of the very enlarged nuclei throughout the 3 segments of proximal tubule, while the tumors arise only in the 1st segment, suggests that they have little relevance to the development of this neoplasm. Further support for this conclusion comes from studies with aflatoxin. Although aflatoxin B1 in single dose induces widespread karyomegaly in proximal tubules (4), subsequent development of renal adenocarcinomas has not been seen (W. H. Butler, unpublished observations). A single injection of aflatoxin G1 also produces widespread renal karyomegaly, although renal tumors have as yet only been produced by long-term feeding (5, 6). The liver also responds to DMN and other carcinogens in this way. However, a stepwise increase in ploidy in DMN-treated rat liver related to the development of enlarged nuclei bears no relationship to cancer (8).

The results indicated that at least some DMN-induced renal adenocarcinomas take their origin from the 1st segment of proximal tubules. Further, the cell type susceptible to neoplastic development is injured in the acute phase of intoxication by the carcinogen. However, we are unable as yet to identify the cellular alterations specific to the early transformation. The use of a system yielding an incidence of renal adenocarcinomas higher than 30% may be necessary to provide such information.

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REFERENCES

Fig. 1. Electron micrograph of proximal tubule showing basal accumulation of lipid droplets (Li) 3 days after DMN treatment. The basement membrane (arrowheads) is normal in appearance. ×12,600.

Fig. 2. Electron micrograph of apical region of proximal tubule at 4 days showing early formation of cytosegresomes (Cs). AV, apical vacuole. ×26,000. Inset, well-formed cytosegresome. ×16,250.

Fig. 3. Electron micrograph of basal cytoplasmic protuberance from damaged proximal tubule at 4 days. The process contains dense bodies, vesicles, and endoplasmic reticulum. The basement membrane (BM) is thickened and condensed into several layers. Basal interdigitations are absent from the cell but bundles of microfilaments (MF) border the basal plasma membrane including that of the protuberance. In the intertubular space, processes of a cortical fibrocyte (CF) and macrophage (Mp) are present. ×19,300.

Fig. 4. Electron micrograph of a small, irregularly shaped process arising from the base of a damaged proximal tubule cell at 7 days. It is joined to the tubule cell by a thin neck of cytoplasm. A smaller process (arrowhead) is also present. The surrounding basement membrane (BM) is thickened and tortuous. In addition to the cytoplasmic process, the intervening space contains membrane-bound and free, clear vesicles and dense granules. A macrophage (Mp) with characteristic fenestrated border lies beside the basal lesion. Lipid droplets (Li) are present in the damaged tubule cell. Basal interdigitations are absent. ×10,500.

Fig. 5. Electron micrograph of basal region of a damaged proximal tubule at 7 days. A tortuous, thickened loop of basement membrane (BM) contains abundant clear and dense granules, vesicles, and membranes. Frequent lipid droplets (Li) are present at the base of the tubule cell. Fenestrated processes of macrophages (Mp) in the intertubular space surround the lesion. ×11,000.

Fig. 6. Electron micrograph of base of damaged proximal tubule cell at 2 weeks. Within the space surrounded by the tortuous, thickened loop of basement membrane (BM) is the fenestrated process of a macrophage (Mp). Phagocytic vacuoles are present in the cytoplasm of this cell. A band of microfilaments (MF) borders the basal plasma membrane of the damaged tubule cell, which is devoid of infoldings. CF, process of cortical fibrocyte; Tb, undamaged tubule. ×15,850.

Fig. 7. Phase-contrast light micrograph of cortex at 7 days. The intertubular space is diffusely infiltrated with mononuclear cells, the predominant type being the macrophage. One proximal tubule contains 2 necrotic cells (arrowheads). ×550.

Fig. 8. Light micrograph of cortex at 7 days. Two cells within proximal tubules immediately located next to a glomerulus (G) are in mitosis (arrowheads). H & E, ×550.

Fig. 9. Light micrograph of cortex at 6 weeks. One proximal tubule contains a grossly enlarged nucleus (N). H & E, ×550.

Fig. 10. Electron micrograph of proximal tubule cell filled with abnormal mitochondria (M) at 12 weeks. They are very enlarged and dense with tubular cristae. Some are ring-shaped. Cortical fibrocytes (CF) and lymphocytes (Ly) are present in the intertubular space. ×4,650.

Fig. 11. Phase-contrast light micrograph of a proliferating tubule (Tb) located beside a glomerulus (G) at 12 weeks. Lesions of this nature were seen as early as 6 weeks and were always associated with glomeruli. ×600.

Fig. 12. Light micrograph of early proliferating tubule(s) at 14 weeks. A mild interstitial cell increase is associated with the lesion. Arrow, an arteriole, indicating close proximity to a glomerulus. The area indicated by the arrowhead is enlarged in the inset to show a cell in mitosis (inset, ×440). H & E, ×190.

Fig. 13. Electron micrograph survey of a lesion at 20 weeks identical to that shown in Fig. 11. One poorly differentiated cell is in mitosis. Some nuclei lack chromat in condensation, and nucleoli are prominent. Brush border (BB) is abnormally located between cells near the periphery of the lesion. The proliferating tubule is surrounded by intact basement membrane (arrowheads). ×3,600.

Fig. 14. Higher magnification of cells within the lesion shown in Fig. 13. Brush border (BB) is shared between 3 peripheral cells. One poorly differentiated cell contains a hypertrophied Golgi apparatus (GA). The basement membrane (BM) is thickened on this side of the lesion. ×7,500.

Fig. 15. Phase-contrast light micrograph of proliferative tubule at 36 weeks. The lesion is located next to a glomerulus and consists predominantly of swollen, clear cells which stain faintly positive with PAS. ×220.

Fig. 16. Electron micrograph of cells within lesion shown in Fig. 15. Much of the cytoplasm is filled with granular material (g). One cell is binucleate, and nucleoli are prominent. Low microvilli similar to those seen in distal tubules line narrow, intercellular spaces (arrowheads). Interdigitations of plasma membrane are seen in the binucleate cell (arrow). ×6,500.
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