Short Communication: Renal Tubular Vacuolation in Animals Treated with Polyethylene-Glycol-Conjugated Proteins

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During toxicologic evaluation of a dimeric PEG-linked protein, tumor necrosis factor binding protein (TNF-bp), vacuolation of renal cortical tubular epithelium was seen in male and female Sprague-Dawley rats (200-300 g) given iv doses of 40, 20, or 10 mg/kg every other day for 3 months. Tubular lesions in rats treated with 20 or 40 mg/kg for 3 months were only partially reversible after a 2-month recovery period. Despite the presence of marked vacuolation, there were no changes in BUN, creatinine, urinalysis parameters, urinary NAG, urinary β2-microglobulin, or fractional sodium excretion. Single iv doses ≥20 mg/kg TNF-bp caused similar but milder changes. However, equivalent doses of PEG alone or the non-PEG-linked TNF-bp did not cause light microscopic evidence of vacuolation. Treatment of rats with another PEG-linked protein of similar molecular weight resulted in similar changes. Immunostaining for TNF-bp revealed positivity in the apical cytoplasm of renal tubular epithelium within 1 h of iv dosing. Immunostaining of kidneys from chronically dosed rats indicated that protein was present in some vacuoles as long as dosing continued; however, kidneys from animals on a reversibility study had vacuoles but no immunostaining for TNF-bp. These results, along with a study that showed more severe lesions with PEG-linked proteins of lower molecular weight and minimal if any lesions with PEG-linked proteins >70 kDa, suggest that TNF-bp is filtered through the glomerulus and that the protein with attached PEG is reabsorbed by the proximal tubules. Vacuolation may be a result of fluid distension of lysosomes due to the hygroscopic nature of PEG. These studies demonstrated that PEG-linked proteins have the capacity to induce renal tubular vacuolation at high doses. However, the change was not associated with alteration of clinical pathology or functional markers. © 1998 Society of Toxicology.

METHODS

Animals

Male and female Sprague-Dawley rats (weighing approximately 200-300 g) from Charles River were used in all studies. Rats were acclimated at least 48 h prior to use. All animal use was in accordance with USDA guidelines for humane care.
RENAL VACUOLATION WITH PEG-LINKED PROTEINS

Test Chemicals; Test Articles

PEGs. Polyethylene glycol 20 kDa bis-vinyl sulfone (PEG bis-vinyl sulfone) was produced from 20-kDa PEG (PEG diol) (Shearwater Polymers, Huntsville, AL). PEG 50 kDa bis vinyl sulfone was produced from 50-kDa PEG diol. Methoxy polyethylene glycol (mPEG) 20 kDa vinylsulfone and aldehyde were produced at Amgen and Shearwater Polymers, respectively, from methoxypolyethylene glycol. The 20-kDa PEG diol used for the rat studies was also purchased from Shearwater Polymers.

Proteins. Cysteine muteins of TNF-bp and IGF-bp1 were made by site-directed mutagenesis. These muteins were produced in Escherichia coli, refolded, and purified.

Preparation of 20-kDa and 50-kDa PEG TNF-bp dimers. The purified TNF-bp cysteine mutein was reacted through the free cysteine residue with PEG 20 kDa or 50 kDa bis vinyl sulfone to produce a TNF-bp PEGylated dimer. The PEGylated dimer was then purified to >95% purity, concentrated, and dialyzed into phosphate buffered saline (MWTs = 56 and 86 K, respectively).

Preparation of TNF-bp and IGF-bp1 PEG monomers. The TNF-bp mutein was reacted through the free cysteine residue with mPEG 20 kDa vinyl sulfone to make a TNF-bp 20-kDa PEGylated monomer. TNF-bp was also reacted with an excess of PEG 50 kDa bis vinyl sulfone to make predominantly TNF-bp 50-kDa PEG monomer. The unreacted vinyl sulfone moiety on the opposite end of the PEG was then reacted with 2-mercaptoethanol to prevent it from binding another TNF-bp molecule or other proteins in vivo. These proteins were purified to >95% purity, concentrated, and dialyzed into phosphate buffered saline (MWTs = 38 and 68 K for TNF-bp 20-kDa monomer and TNF-bp 50-kDa monomer). The IGF-bp1 mutein was reacted through the free cysteine residue with mPEG 20 kDa maleimide (Shearwater Polymers) to make an IGF-bp1 20-kDa PEG-linked monomer. This protein was also purified to >95% purity, concentrated, and dialyzed into phosphate buffered saline (MWT = 43 K).

Clinical Pathology

The following clinical chemistry parameters were determined (Ciba-Corning Express Plus Automated analyzer) after 1 or 3 months of dosing with TNF-bp in the chronic reversibility study: blood urea nitrogen, creatinine, total protein, globulin, sodium, potassium, calcium, lactate dehydrogenase, chloride, and phosphorus.

Histopathologic Evaluation

One-half of one kidney was collected into 10% formalin and processed for light microscopic evaluation of vacuolar change. The other half was collected for frozen sections for oil red O fat staining. The second kidney was collected into 4% paraformaldehyde for immunostaining for TNF-bp. Immunostaining was done with a goat antibody (Amgen Boulder, 2.0 mg/ml) diluted 1:500 and an immunoperoxidase procedure (VectaStain, Burlingame, CA). An irrelevant mouse antibody (anti-IL-1ra) was used at equal concentrations as a negative control. The chronic study, scoring of renal tubular vacuolation was based on the following criteria:

Minimal—very few vacuoles evident; if present, generally less than 4 μm in diameter.

Mild—vacuoles clearly evident with individual epithelial cells generally containing one to several vacuoles with a maximum diameter of 7 μm.

Moderate—vacuolar change clearly evident at lower magnifications (100X total) because of clustering of the lesion, greater number of large vacuoles (7 μm) but very little coalescence to multicellular morphology. No significant distortion of tubular profiles due to enlargement of epithelial cells.

Marked—vacuolar change clearly evident at 16X total magnification, large areas of cortex affected, tubules extremely distorted due to presence of very large (up to 30 μm in diameter) vacuoles which often coalesced to form vacuolar structures with poorly defined boundaries, marked distortion of tubular profiles, nuclei compressed, and sometimes pyknotic appearing.

Severe—similar to marked but involving more cortical surface area.

Since the lesions were milder in the studies in which only one or two doses of TNF-bp were given, it was necessary to use a different scoring system to differentiate lesion severity in the dose groups. Therefore, in these studies minimal lesions consisted of very few vacuoles generally <3 μm in diameter. Mild lesions consisted of clearly discernible vacuoles which were generally 3 μm in diameter; moderate lesions were those in which the vacuoles were >3 but <7 μm in diameter and marked lesions were those in which the vacuoles were of similar size to those described under moderate but affected more tubules.

Chronic Dosing of TNF-bp: Evaluation of Renal Vacuolation and Its Potential Reversibility

Female rats (11/group) were treated every other day for 1 or 3 months with 4, 10, 20, or 40 mg/kg TNF-bp iv or vehicle (5 ml/kg). Three rats/group were killed after 1 month of dosing. Five rats/group were killed after 3 months of dosing. Three rats/group were killed after 3 months of dosing and a 2-month recovery period in which no dosing was done. Kidneys were collected for frozen sections for oil red O fat staining. The other half was collected for light microscopic evaluation of vacuolar change. The other half was collected into 10% formalin and processed for routine urinalysis, urine-NAG, and urine B2-microglobulin. Serum samples were also collected at the end of the 22-h period so that fractional excretion of sodium could be calculated by the following formula: fractional excretion of sodium = 100X (urine Na/plasma Na) divided by (urine creat/plasma creat.)

Single Dose of TNF-bp: Evaluation of Renal Vacuolation

Male rats (3/group) were given single iv doses of 20 or 40 mg/kg TNF-bp or non-PEG TNF-bp and then killed 48 h later to evaluate light microscopic renal tubular vacuolation. In addition, male rats (3/group) were given single iv doses of 3 mg/kg TNF-bp and then killed 1 or 3 h postdosing to evaluate immunostaining for TNF-bp in epithelial cells of the proximal tubules.

Comparison of PEG Alone vs PEG-Linked Proteins on Renal Vacuolation

Male rats (3/group) were given two iv 40 mg/kg doses (48-h intervals) of TNF-bp, IGF-bp1, PEG vinyl sulfone, PEG diol, ethylene glycol, or vehicle and then killed 48 h after the second dose to evaluate light microscopic renal tubular vacuolation. This study evaluated the capacity of PEG-linked proteins as well as intermediates involved in the synthesis of PEG-linked proteins to produce tubular vacuolation. In addition, ethylene glycol was tested in the same dosing paradigm.

Comparison of Different Molecular Weight Constructs of PEG-Linked Proteins on Renal Vacuolation

In an attempt to determine if lower molecular weight constructs of PEG-linked proteins would be more readily filtered through the glomerulus and thus produce greater tubular vacuolation, the following proteins were administered iv (two doses, 40 mg/kg, 48-h intervals). Male rats (4/group) were treated with TNF-bp 20-K PEG dimer (MW = 56.3 kDa), TNF-bp 20-K PEG monomer (MW = 38 kDa), TNF-bp 50-K PEG dimer (MW = 86 kDa), and TNF-bp 50-K PEG monomer (MW = 68 kDa). Rats were killed 48 h after the second dose and kidneys collected.
TABLE 1
Incidence and Severity of Treatment-Related Renal Tubular Vacuolar Lesions in Rats Treated Every Other Day with TNF-bp for 1 Month (a) or 3 Months (b)

<table>
<thead>
<tr>
<th>Histopath grade</th>
<th>1a</th>
<th>1b</th>
<th>2a</th>
<th>2b</th>
<th>3a</th>
<th>3b</th>
<th>4a</th>
<th>4b</th>
<th>5a</th>
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<tr>
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<td>3</td>
<td>5</td>
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<td>5</td>
<td>3</td>
<td>5</td>
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<td>5</td>
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<td>3</td>
<td>5</td>
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</table>

Note: Vacuolation scores ranging from 0-4 were given with 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked.

RESULTS

Effects of Chronic Dosing of TNF-bp on Renal Vacuolation and Evaluation of Potential Reversibility

Clear, variably sized vacuoles were distributed throughout the cytoplasm of epithelial cells lining the proximal tubules of animals in the 10, 20, and 40 mg/kg dose groups in the studies (1 or 3 months duration of dosing) in which animals were killed immediately following cessation of treatment (Table 1, Fig. 1). Fat stains for neutral lipid were negative.

Kidneys from rats dosed for 3 months and allowed 2 months of recovery had lesions of reduced severity compared to their counterparts in the 3-month study. Rats treated with 10 mg/kg and killed 2 months after the last dose had no tubular vacuolation compared to those killed after 3 months, which had minimal to moderate changes. One rat treated with 20 mg/kg and allowed to recover had moderate vacuolation whereas the other two had no evidence of tubular vacuolation. All rats treated with 40 mg/kg had moderate to marked vacuolar change despite the 2-month recovery period. However, there was obviously some repair as those treated for 3 months without recovery generally had severe change. In comparing the morphology of the vacuolar change in the rats treated for 3 months to those treated for 3 months with a 2-month recovery, it was evident that, in general, those allowed to recover had retention of large vacuoles whereas those treated for 3 months and then immediately killed had a mixture of large and small vacuoles in the cytoplasm of the tubular epithelium. Thus, it seemed that the smaller vacuoles repaired but the larger ones did not. Immunostaining results for protein (TNF-bp) revealed abundant positivity in rats dosed for 3 months and then killed. Positive staining occurred only in vacuolated tubules and generally was characterized by rimming of the vacuoles rather than central or diffuse staining. Rats dosed for 3 months and then allowed a 2-month recovery period without dosing had no positive staining for TNF-bp.

There were no abnormalities in clinical pathology parameters, fractional excretion of sodium, urine NAG, or urine B2-microglobulin (data not shown).

Effects of a Single Dose of TNF-bp on Renal Vacuolation

Male rats given single 20 mg/kg doses had minimal (1) or mild (2) lesions, while all rats given 40 mg/kg had moderate change. There were no lesions in rats treated with 4 mg/kg. Immunostaining for TNF-bp was minimal or negative in those given 4 mg/kg TNF-bp. However, those treated with 20 or 40 mg/kg had definite staining of vacuoles. Rats given single iv doses of 3 mg/kg and then killed 1 or 3 h later for evaluation of immunostaining patterns of TNF-bp in kidney had evidence of staining in apical lysosomes and brush border of tubular epithelial cells with the intensity being greater at 3 h than at 1 h. Rats treated with 40 mg/kg non-PEG TNF-bp had no lesions and no immunopositivity 48 h postdosing.

Comparison of PEG Alone vs PEG-Linked Proteins on Renal Vacuolation

Male rats (3/group) given two iv 40 mg/kg doses (48-h intervals) of TNF-bp or IGF-bp1 had minimal to mild vacuolation of renal tubular epithelium. Those treated with similar doses of PEG vinyl sulfone, PEG diol, ethylene glycol, or vehicle had no microscopic alterations.

Comparison of Different Molecular Weight Constructs of PEGylated Protein on Renal Vacuolation

Dosing rats with different molecular weight constructs of PEG-linked protein clearly demonstrated differences in lesion severity that were dependent on total molecular weight. Animals treated with the lowest molecular weight protein
had the most severe vacuolar changes while those treated with the higher molecular weight material had minimal if any lesions (Table 2).

**DISCUSSION**

Results of these studies demonstrate that PEG-linked proteins with molecular weights less than 70 kDa have the capacity to induce marked renal cortical tubular vacuolation in animals given sufficiently high doses. The lesion can be induced with single 20–40 mg/kg iv doses. In these animals, the vacuoles are small and discreetly singular and do not distort the epithelial cells or compress nuclei. Repetitive dosing of similar high doses results in formation of large single or coalescing vacuoles which distort tubular profiles and compress nuclei. With chronic dosing, vacuolation becomes evident at doses (10 mg/kg) that do not cause lesions when single doses are given. Mild lesions are reversible while those which have progressed to the multilocular stage are not reversible after 2 months without dosing. Although it has been shown that exposure to PEG alone can result in ultrastructural vacuolation of renal proximal tubules (Alden and Frith, 1991), we never saw light microscopic evidence of vacuolation with PEG doses slightly greater (2×) than those given when the combination PEG–protein was administered. Since exposure to the non-PEG-linked monomeric protein (TNF-soluble receptor) did not induce vacuolation either, we concluded that the protein alone was not responsible for the effect. Also, since dosing with another PEG-linked protein, IGF-hp1, resulted in similar lesions it seemed likely that the combination of PEG and protein was necessary to induce changes of sufficient magnitude to be seen by light microscopy.

Our immunohistochemical results showing uptake of TNF-hp by proximal tubular epithelial cells within an hour of dosing clearly demonstrate that this 56.3-kDa PEG-linked protein crosses the glomerulus and is probably reabsorbed
by these cells. The finding of TNF-bp immunoreactivity in chronically dosed animals with vacuolar change supports this hypothesis. Animals dosed for 3 months and then killed after 2 months without dosing (reversibility study) retained vacuolation; however, immunostaining was negative. These results suggest that lysosomal proteases are able to process the protein but are unable to deal with the PEG. Breakdown of PEG would theoretically require an enzyme capable of attacking ether linkages (etherase) and this is not a common enzyme in mammalian systems.

Our proposed pathogenesis of this lesion is that the TNF-bp (56.3 kDa) is slowly filtered by the glomerulus. The protein with attached PEG is then pinocytosed by the proximal tubules into apical lysosomes. Lysosomal enzymes efficiently process the protein but not the PEG. When high single doses or repetitive lower doses are given, the hydroscopic PEG accumulates and results in lysosomal distension and epithelial cell distortion. With time, some lesion reversal occurs as a result of epithelial cell turnover. However, we saw no evidence of enhanced cell proliferation with proliferative tubular vacuolation in laboratory animals given appropriately high doses. These lesions were detectable only by histologic evaluation of the tissue, as various clinical chemistry parameters remained normal throughout the evolution of the lesion. The clinical significance of this lesion is currently unknown.

### REFERENCES


