Metabolic Substrates Alter Attachment and Differentiated Functions of Proximal Tubule Cell Culture[1]

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ABSTRACT

Proximal tubules cultured *in vitro* gradually lose their differentiated functions. Because standard culture media lacks several substrates important for renal proximal tubule oxidative metabolism, whether a mixture of substrates including butyrate, alanine, and lactate (BAL) would modify growth and/or differentiated function of proximal tubular cells in culture was examined. Tubules cultured in media supplemented with 2 mM butyrate, alanine, and lactate exhibited enhanced attachment but did not exhibit an altered growth rate. Higher levels of phosphoenolpyruvate carboxykinase and leucine-amino peptidase were sustained, although these activities were still diminished in comparison with that in fresh tubules. Sodium-dependent glucose uptake and dome formation—other reflections of epithelial cell differentiated function—also were enhanced. These studies demonstrate that the substrates used to culture proximal tubules can modify both their attachment and their manifestation of differentiated function in culture.

Key Words: *glucose transport, phosphoenolpyruvate carboxykinase, butyrate, leucine-amino peptidase*

Renal proximal tubules have been grown in primary culture by several laboratories using serum-free hormonally defined media (1–5). However, a variety of technical problems have been noted, including low seeding efficiency, retarded cellular growth, and loss of specific proximal tubular functions. Cultured proximal tubules gradually lose activities of their brush border enzymes, leucine-amino peptidase (LAP), and γ-glutamyl transpeptidase and exhibit reduced Na-dependent glucose transport, reduced mitochondrial capacity for oxidative metabolism, and loss of their gluconeogenic capacity (4,5). Lack of extracellular matrix, growth factor(s), nutrition, or a three-dimensional structure in conventional culture systems all potentially could account for the alteration of growth and differentiation of cultured cells (6,7).

Proximal tubular cells, which are gluconeogenic *in vivo* (8), rely primarily on mitochondrial oxidation for energy (9,10). We previously found that provision of adequate oxygen enhanced dome formation by cultured proximal tubule cells but had no significant effect on either LAP or the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and fructose 1,6 bisphosphatase (4). A variety of substrates including amino acids, fatty acids, and glucose metabolites are important fuels for proximal tubules (9,11). In rabbit proximal tubules, butyrate, alanine, and lactate (BAL) in combination produce the maximal rate of basal and ADP-stimulated oxygen consumption (12,13). However, standard culture media, for example, Dulbecco's modified Eagle's medium and Ham F-12, are deficient in these substrates. Because the lack of gluconeogenic and/or oxidative substrates might impair the behavior of cultured proximal tubules, we supplemented the standard culture media with BAL and assessed growth, attachment, and several characteristics of proximal tubular cell differentiation.

METHODS

Our methodology for primary rabbit proximal tubular isolation and culture has been described previously (4,5). Aliquots of proximal tubule suspension were inoculated onto 60- or 100-mm Corning dishes (Corning Signs Product, Corning, NY) and the culture media were changed 1 day after seeding and then every other day. The control culture medium was 1:1 HAM F-12 base and Dulbecco's modified Eagle's medium with glutamine 2 mM, supplemented with penicillin (192 IU/mL), streptomycin (200 μg/mL), insulin (5 μg/mL), hydrocortisone (5×10^{-8} M), and transferrin (5 μg/mL). BAL (0.5 to 2.0 mM) in combination or other substrates were added to the experimental culture media as indicated.

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Tubule attachment was determined by dividing the protein content of attached tubules (1 day after plating the tubules in the culture) by the total protein content of inoculated tubules. The other analytical methods used by our laboratory have been described previously (4,5).

Statistics were performed by the use of paired or nonpaired t tests and analysis of variance to assess multiple comparisons. Statistical significance was considered at $P < 0.05$.

RESULTS

Effect of BAL on Tubule Attachment and Growth

BAL supplementation of the culture media facilitated tubule attachment in a dose-dependent fashion (Figure 1). When the media was supplemented with BAL (0.5 or 2 mM) at the initiation of culture, enhanced attachment resulted in significantly higher protein levels at Day 4 (during the proliferative phase) but no significant difference on Day 6 (during confluence). This difference in protein levels on Day 4 was abolished if the media were changed to BAL-supplemented media after the first day in culture, confirming that BAL enhanced tubule attachment but did not accelerate growth rate.

When fresh proximal tubules were incubated in media supplemented with 10 mM BAL (10 mM butyrate, 2 mM alanine, and 2 mM lactate), they did not exhibit evidence of proliferation, despite improved attachment. Cells migrated out of tubules onto the culture plate but did not grow.

Effect on BAL of Tubular Differentiation

Four parameters of functional differentiation of proximal tubular epithelium were assessed: dome formation, PEPCK activity, LAP activity, and sodium-dependent glucose transport.

As shown in Table 1, BAL increased dome formation, LAP activity, and sodium-dependent glucose transport in a dose-dependent fashion in comparison with control media. However, the LAP activity, even with 10 mM BAL, was lower than in freshly isolated tubules. PEPCK activity, measured on both Days 4 and 6 in culture, was higher in BAL-supplemented media in a dose-dependent fashion but was decreased in comparison with the activity in freshly isolated tubules (Figure 2).

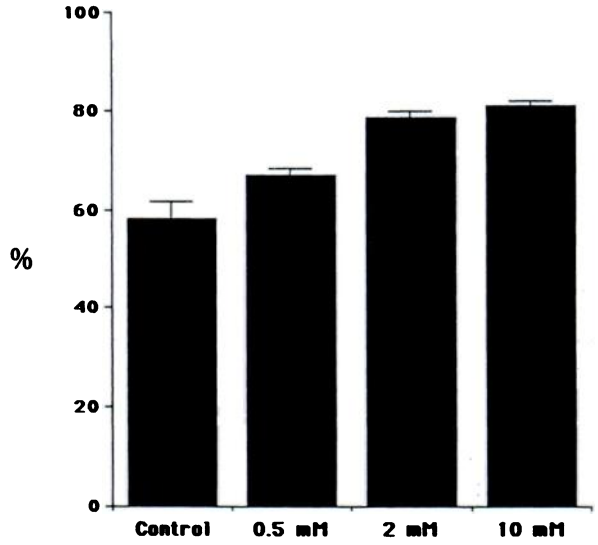

Figure 1. Tubule attachment of primary culture of rabbit proximal tubules in standard (control) and BAL-supplemented media. Each bar represents the mean ± SE of six samples. 0.5 and 2 mM BAL stands for the addition of concentrations of 0.5 and 2 mM of each substrate to the culture media, whereas 10 mM BAL stands for 10 mM butyrate, 2 mM alanine, and 2 mM lactate. The attachment of cultures are in the following order: control < 0.5 mM < 2 mM < 10 mM, ($P < 0.05$, 0.5 mM versus control; $P < 0.01$, 2 versus 0.5 mM).

Substrate-Specific Differentiated Function

We also examined the effects of culture media supplementation individually with 2 mM butyrate, alanine, pyruvate, and α-ketoglutarate. Tubule attach-

TABLE 1. Effects of BAL on differentiation[a]

	Control	BAL		
		0.5 mM	2.0 mM	10.0 mM
Dome Formation (Number/10 Fields)	4 ± 2	7.8 ± 4[b]	18.9 ± 2[c]	
LAP Activity[d] (U/mg)	6.7 ± 0.7	8.8 ± 1.3	10.1 ± 0.5[b]	17.9 ± 0.7[b]
Na^+-Dependent Glucose Transport (nmol/mg per 30 min)	7.8 ± 0.2		15.6 ± 1.0[b]	29.4 ± 2.4[b]

[a] All parameters were assessed on Day 6 in culture.
[b] $P < 0.05$ versus control.
[c] $P < 0.01$ versus control.
[d] LAP activity in freshly isolated tubules averaged 22 ± 2.1 U/mg.

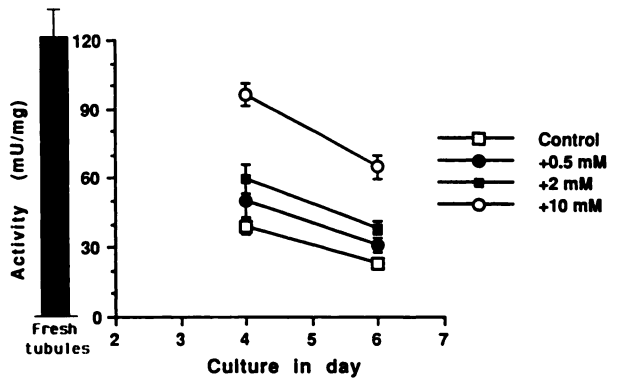

Figure 2. PEPCK activity in primary culture of rabbit proximal tubules in standard (control) and BAL-supplemented media. Each point represents mean ± SE of six samples for control, 0.5, and 2.0 mM and four samples for 10 mM BAL. PEPCK activity, which averages 120 + 12 mU/mg in fresh tubules, gradually decreased in control cultures and was up-regulated by BAL in a dose-responsive fashion. Analysis by analysis of variance indicated a significant difference for all comparisons at Day 6 with the exception of 2.0 versus 0.5 mM BAL.

ment was increased significantly only by alanine and α-ketoglutarate alone. PEPCK activity and LAP activity were increased only by butyrate. Sodium-dependent glucose transport was higher with all four substrates individually, but the increase was not statistically significant with butyrate.

DISCUSSION

Studies of the metabolic substrates required for cell growth in tissue culture were rigorously pursued in the 1960s (14). Defined culture media, which were designed initially to support the culture of mesenchymal cells, subsequently were found to be suitable for culture of epithelial cells, including renal proximal tubules (14,15). However, these media do not provide several substrates that are important fuels for proximal tubules. On the basis of the evidence that a combination of butyrate, alanine, and lactate supported the highest basal oxidative metabolic rates in freshly isolated rabbit proximal tubules, we decided to examine the effect of this combination of substrates on growth and several differentiated functions of cultured proximal tubules (4,5,12,13).

As shown in Figure 1, BAL supplementation resulted in a marked increase of tubular attachment. In our hands, the attachment on plastic dishes with 2 mM BAL-supplemented media is markedly higher than with 10% fetal calf serum or when collagen-coated dishes are used (data not shown). The improved attachment does not appear to be dependent on the capacity of the substrate for oxidative metabolism, because both α-ketoglutarate and alanine alone dramatically enhanced attachment, whereas pyruvate, which can be oxidized as efficiently as alanine, had no effect on attachment. Furthermore, butyrate alone did not significantly enhance attachment. Although the precise mechanism whereby specific substrates enhance attachment is unexplained, BAL supplementation provides a useful tool to improve the technique of primary proximal tubular cell culture.

Dedifferentiation of cells grown in culture has been widely recognized, but the mechanism accounting for this process is not understood. We previously found that the dedifferentiation process in primary cultures of proximal tubules is manifested by reversion to glycolytic metabolism and the gradual loss of several differentiated functions including gluconeogenesis, brush border enzyme (LAP and γ-glutamyl transpeptidase) activity, and Na-dependent glucose transport (4). Because a precise marker of the differentiated phenotype of proximal tubule cells is unavailable, it has been necessary to rely on a variety of functional markers to assess potential alterations in the differentiation of these cells.

These studies demonstrate that substrates available in the culture media can modulate the differentiated function of proliferating cells. We found that BAL supplementation of the media, in concentrations that did not alter the rate of growth (0.5 to 2 mM), resulted in the maintenance of higher PEPCK levels. In addition, higher activity of the brush border enzyme LAP, an increased rate of Na-dependent glucose transport, and enhanced dome formation were found in cultures supplemented with BAL. Thus, this combination of substrates, in some fashion, resulted in cells with better, although still not normally preserved, features of differentiated function. Furthermore, 10 mM BAL, which strikingly inhibited growth, increased PEPCK, LAP, and sodium dependent glucose transport substantially above the values observed with 2 mM BAL.

The individual substrates and the precise mechanisms responsible for the effects of BAL on the various differentiated functions represent a complex issue. Clearly, the effects on LAP and PEPCK activity are a distinct effect of butyrate, because no other individual substrate tested exhibited any effect on these functions. However, virtually every substrate examined appeared to up-regulate the capacity for sodium-dependent glucose transport, although the mean increase observed with butyrate alone did not achieve statistical significance.

The effect of butyrate on cell morphology, growth, and differentiation has been investigated in many cell lines (16–20). Butyrate, at an 8 mM pharmacologic dose, stimulates gene expression and cell differentiation via increasing histone acetylation and also inhibiting histone deacylation. However, in several cultured cell systems, concentrations of approximately 2.0 mM induce differentiated features (18,19)

and effects on differentiation have even been noted with concentrations as low as 0.1 mM (20). We found that 2 mM butyrate, a concentration that does not alter growth, was sufficient to increase activities of the gluconeogenic enzyme PEPCK in cultured proximal tubules. Furthermore, we also found that 0.5 mM BAL produced significant, up-regulation of PEPCK activity and also enhanced dome formation. Because the butyrate concentration in serum has not been reported, it is not possible to conclude with certainty whether these effects are mediated by pharmacologic or physiologic action. Nevertheless, the low concentrations of butyrate that exhibit effects on cultured proximal tubule cells are suggestive of a mechanism related to the substrate properties of butyrate. Of interest, others have found that supplementation with the longer chain fatty acid heptanoate has no effect on the down-regulation of the gluconeogenic enzyme fructose-1,6-bisphosphatase or on the brush border enzyme alkaline phosphatase (21).

We conclude that the standard media currently used for renal cell cultures are not optimal and that BAL supplementation results in the maintenance of improved differentiated functions by cultured proximal tubules. BAL enhances dome formation, stimulates gluconeogenic and brush border enzyme activity, and also up-regulates Na-dependent glucose uptake, all in a dose-responsive fashion. Substrate supplementation also enhances the attachment of cultured proximal tubules and thereby confers an additional experimental advantage. Supplementation of the culture media in this fashion thereby is useful for improving the utility of this system as a model for behavior of cells *in vivo*.

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