Suppression of Dysplasia and Hyperplasia by Calcium in Organ-cultured Urinary Bladder Epithelium

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ABSTRACT

The cause for the development of endophytic growth (nodular downgrowth) in rat urinary bladder epithelium cultured in protein-free medium was traced to a deficiency of Ca\(^{2+}\) in the culture medium. Endophytic growth, which is a characteristic in vivo histological feature of the pre-neoplastic phase of experimental bladder cancer, was induced in vitro by 0.3 mM Ca\(^{2+}\) but was suppressed by 1.8 mM Ca\(^{2+}\). Magnesium at 1.8 mM had no effect on endophytic growth.

Ca\(^{2+}\) deficiency was also found to be a significant cause of hyperplasia. Ca\(^{2+}\) concentrations above 0.9 mM resulted in a substantial inhibition of hyperplasia in the bladder epithelium; a greater than 50% inhibition was observed with 1.8 mM Ca\(^{2+}\). Conditions were found, however, which caused the suppression of endophytic growth without an apparent suppression of hyperplasia.

When the Ca\(^{2+}\) concentration was decreased to 0.075 mM and below, epithelial cells lost cohesiveness and infiltrated into the stroma. Epithelial cells were observed individually and in clusters within the lamina propria. Evidence of lytic activity was also observed in the lamina propria. Epithelial cells that infiltrated into the stroma displayed occasional mitotic figures and frequently contained abnormally appearing nucleoli.

The ability of low Ca\(^{2+}\) concentration in the culture medium to induce epithelium to mimic some of the growth patterns that appear during the development of experimental bladder cancer in vivo, suggests that there may be an early change in some aspect of cellular Ca\(^{2+}\) in epithelium and/or stroma that have been exposed to bladder carcinogens.

INTRODUCTION

In earlier studies (16, 17) we demonstrated that the urinary bladder epithelium of the rat can be cultured in various media without the addition of serum or other protein supplements. In MEM-EBS\(^1\) and Waymouth's MB752/1 medium, organ-cultured epithelia proliferated but retained their characteristic transitional architecture. In Ham's F12 medium, on the other hand, extensive proliferation and dysplasia were observed. The most prominent dysplastic feature of bladder epithelia cultured in F12 medium was the development of endophytic growth (nodular downgrowth) into the underlying stroma. This response of bladder epithelia to F12 medium is especially interesting because the development of endophytic growth is a characteristic feature of preneoplastic bladder epithelia that have been exposed to known bladder carcinogens (2, 14, 19). Endophytic growth has been stated to be the most important histological feature of carcinoma in situ in bladders of rats that received the bladder-specific carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine in the drinking water (14). It is not known, however, whether endophytic growth should be considered a precursor lesion to cancer, or whether it should be considered merely a manifestation of an unstable epithelium that may or may not eventually have a direct relationship to the development of cancer (see Refs. 5, 13, and 19). From an experimental point, we think that the important aspect of endophytic growth, whether it is induced by carcinogen or is induced in vitro by F12 medium, is that it represents a breakdown in the normal architecture of the epithelium in which cells have extended beyond the territories that they normally occupy. Since invasive cancer is an extreme example of cells that have extended beyond their normal territorial boundaries, it would be of considerable significance to know why endophytic growth arises in the bladder epithelium and how this particular pattern of growth can be suppressed.

In this paper we present an analysis of endophytic growth in F12 medium and identify the cause of this growth to be a deficiency of Ca\(^{2+}\) in the culture medium. We also demonstrate that Ca\(^{2+}\) deficiency is a significant cause of the hyperplastic response in organ-cultured bladder epithelium.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats were obtained from the Charles River Breeding Laboratories, Wilmington, Mass., and used at 7 weeks of age.

Organ Culture Procedure. Animals were anesthetized with CO\(_2\). Urinary bladders were removed aseptically and cut into equal halves longitudinally. Explants were cultured, with the epithelium positioned dorsally, on stainless steel grids in 60-mm plastic culture dishes containing 2 ml of medium. The medium contained 100 units of penicillin and 100 \(\mu\)g of streptomycin per ml. Cultures were maintained at 36° in a gas phase of 4.5% CO\(_2\) in air. Medium was changed every 2 or 3 days. Unless specified differently, all culture media components were obtained from Grand Island Biological Co., Grand Island, N. Y. Calcium chloride and magnesium chloride were reagent grade and obtained from Baker and Adamson and Mallinckrodt, respectively. Within each experiment, 4 to 6 explant cultures were prepared for each specific medium formulation investi-

\(^{1}\) The abbreviations used are: MEM-EBS, Eagle's minimal essential medium with Earle's balanced salt solution; MEM, Eagle's minimal essential medium; EBS, Earle's balanced salt solution.

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RESULTS

Ca\(^{2+}\) Suppresses Endophytic Growth. Our initial approach in attempting to identify the cause of endophytic growth in F12 medium was to investigate whether the elimination of some of the growth-associated components present in F12 medium, such as putrescine and linoleic acid, would eliminate endophytic growth. It was found that, although more growth occurred in medium with putrescine and linoleic acid than in medium without these components, endophytic growth still occurred in their absence.

Rather than test all suspected components individually for their ability to produce endophytic growth, we decided to test the major groups of components such as the salts and amino acids of F12 medium for their ability to induce endophytic growth when substituted for the same components of MEM-EBS which do not normally cause endophytic growth. The first component tested, the salts component, proved to be the cause of endophytic growth. When one-half of a bladder was cultured in MEM-EBS, growth was nonendophytic (Fig. 1A). When the other half of the same bladder was cultured in MEM containing the salts component of F12 medium, endophytic growth occurred (Fig. 1B).

One of the major differences between F12 salts and EBS is the presence of copper, iron, and zinc in F12 salts. These were obtained from 2 sources (Grand Island Biological Co. and Johnson, Matthey and Co., London, England) and were tested in MEM-EBS for their ability to induce endophytic growth. None was effective, either singly or in combination with others, in stimulating endophytic growth.

Another major difference between F12 salts and EBS is in the Ca\(^{2+}\) concentration; F12 salts contain 0.3 mM Ca\(^{2+}\) while EBS contains 1.8 mM. Experiments were carried out to determine whether, by elevating the Ca\(^{2+}\) level in F12 salts, endophytic growth could be blocked. When one-half of a bladder was cultured in MEM with F12 salts, endophytic growth developed (Fig. 2A). Endophytic growth was suppressed, however, in the other half of the same bladder cultured in MEM with F12 salts, in which the Ca\(^{2+}\) concentration had been increased to 1.8 mM (Fig. 2B).

In addition to suppressing endophytic growth, elevated Ca\(^{2+}\) levels also produced a more organized epithelium. Even when compared with nonendophytic areas of epithelia cultured in 0.3 mM Ca\(^{2+}\) (Fig. 3A), epithelia cultured in 1.8 mM Ca\(^{2+}\) (Fig. 3B) had a more ordered cellular arrangement and frequently displayed a more transitional morphology.

Another characteristic of epithelia cultured in high Ca\(^{2+}\) concentration was their reduced affinity for the underlying stroma. Epithelia cultured in 1.8 mM Ca\(^{2+}\) had a tendency, after 14 days of culture, to occasionally separate from the stroma (Fig. 4A). These epithelia displayed a surprising affinity for the stainless steel grid, which resulted in occasional tearing of epithelia during the fixation process. Epithelia cultured in 0.3 mM Ca\(^{2+}\), on the other hand, exhibited a high affinity for the underlying stroma. The epithelial-stromal borders of these epithelia (Fig. 4B) were usually less clearly defined than those of epithelia cultured in 1.8 mM Ca\(^{2+}\).

Another feature of epithelia cultured in 1.8 mM Ca\(^{2+}\), which was revealed most clearly when the epithelium became separated from the stroma as in Fig. 4A, was the presence of extremely flattened cells lining the epithelial-stromal border. These cells were not evident in those endophytic regions of epithelia cultured in 0.3 mM Ca\(^{2+}\), in which the epithelial-stromal borders were not clearly defined (Fig. 4B). Some were observed, however, in endophytic regions in which the epithelial-stromal borders were more clearly defined (Fig. 3A), but these cells were not as compressed.

Experiments were carried out to determine whether, by increasing the magnesium concentration to 1.8 mM in the presence of 0.3 mM Ca\(^{2+}\), endophytic growth could be blocked, or if the elevated magnesium concentration interfered with the suppression of endophytic growth by 1.8 mM Ca\(^{2+}\). Magnesium was ineffective in both instances. Since calcium chloride and magnesium chloride were used in these experiments, these results also indicate that Ca\(^{2+}\), and not Mg\(^{2+}\), suppressed endophytic growth.

Ca\(^{2+}\) Suppresses Hyperplasia. In addition to demonstrating that 1.8 mM Ca\(^{2+}\) suppressed endophytic growth, a comparison of explants cultured in low and high Ca\(^{2+}\) also suggested that high Ca\(^{2+}\) (1.8 mM) suppressed hyperplasia. To verify these observations quantitatively, the extent of hyperplasia was measured, by a previously described method (17), in epithelia that were cultured for 12 days in MEM with F12 salts containing Ca\(^{2+}\) concentrations ranging from 0.3 mM to 1.8 mM. In these experiments, endophytic growth was not as pronounced as that encountered in 14-day cultures and was thus more amenable to quantitation. The suppression of hyperplasia by Ca\(^{2+}\) was dose dependent (Chart 1); a substantial suppression was observed when the Ca\(^{2+}\) concentration was increased above 0.9 mM. In medium containing 1.8 mM Ca\(^{2+}\), the extent of hyperplasia was less than 50% of that occurring in medium containing 0.3 mM Ca\(^{2+}\).

Since higher Ca\(^{2+}\) levels suppressed hyperplasia in addition to endophytic growth, it could be implied that endophytic growth occurs as a direct result of excess hyperplasia. Two lines of evidence demonstrate that this is not necessarily the case. When bladders were cultured in MEM-EBS in which all medium components were in higher concentration (1.4 times) than normal, excess hyperplasia occurred. Epithelia became approximately 10 cell layers thick by 7 days of culture. No endophytic growth developed, however. In another series of experiments, bladders were cultured for 14 days, one half in F12 medium and the other half in F12M medium (B), which contains elevated levels of several components including Ca\(^{2+}\) (1.1 mM). In F12 medium, growth was endophytic (Fig. 5A). In F12M medium no endophytic growth occurred (Fig. 5B) and, although it was not possible quantitatively to compare the actual amount of growth with that which occurred in F12 medium because of the extensive endophytic growth in F12, the extent of growth in the 2 media appeared to be comparable.

Low Ca\(^{2+}\) Concentration Stimulates Epithelial Cell Infiltration Into the Stroma. In view of the organizing effect that Ca\(^{2+}\) exerted on the bladder epithelium in culture, the
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Chart 1. Effect of Ca\textsuperscript{2+} on hyperplasia. Explants were cultured for 12 days in MEM with F12 salts. Each point represents the mean number of nuclei per unit length (150 \( \mu \)m) of epithelia from 4 or 5 explants ± S.E. Hatched area, mean values ± S.E. for normal (uncultured) epithelia.

The results obtained in this study show the Ca\textsuperscript{2+} concentration to be a major determinant of both the quantitative and qualitative aspects of growth of the bladder epithelium in organ culture.

The significant suppression of hyperplasia by Ca\textsuperscript{2+} that we observed is quite interesting, since there have been reports (1, 10, 18, 20) demonstrating that Ca\textsuperscript{2+} stimulates cell proliferation at concentrations that we have found to be inhibitory. A discussion of why this discrepancy should exist would be premature at this point, since there are numerous differences between our experiments and those showing stimulation by Ca\textsuperscript{2+}. Three major points should be emphasized about this study, however. (a) The effect of Ca\textsuperscript{2+} concentration was measured on organ-cultured tissue in serum-free medium. (b) In stating that Ca\textsuperscript{2+} suppressed hyperplasia, we are not necessarily indicating that the rate of cell proliferation was reduced by Ca\textsuperscript{2+}. An epithelium can become hyperplastic in essentially 2 ways, through an increased rate of proliferation or a decreased rate of maturation and cell sloughing from the surface. It is entirely possible, for example, that the rate of proliferation in high Ca\textsuperscript{2+} concentration was equal to or even greater than that in low Ca\textsuperscript{2+} concentration, but that the rate of maturation was greater in high Ca\textsuperscript{2+}. If, in the presence of high Ca\textsuperscript{2+} concentration, the suppression of hyperplasia was due to the inhibition of cell proliferation, then our results can best be interpreted in terms of the energy requirements for cell proliferation (see Ref. 3). Magnesium is a cofactor for the 3 transphosphorylation reactions of glycolysis. Ca\textsuperscript{2+}, because it competes with magnesium for either the required enzyme or the adenine nucleotide of these reactions, is capable of inhibiting the reactions. Thus, it is possible that in the presence of high Ca\textsuperscript{2+} concentration, the bladder epithelium lacked sufficient energy to proliferate at the same rate as that in low Ca\textsuperscript{2+} concentration. (c) In this study the effect of Ca\textsuperscript{2+} was measured on epithelial cells. Previous studies showing Ca\textsuperscript{2+} to be stimulatory to growth were on bone marrow- and thymus-derived cells (20) and fibroblasts (1, 10, 18). It is interesting, in connection with those observations on fibroblasts, that in this study, although Ca\textsuperscript{2+} had an inhibitory effect on the epithelium, stromal cells (presumably fibroblasts) appeared to be stimulated by Ca\textsuperscript{2+} as evidenced by their accumulation at the epithelial-stromal border in high Ca\textsuperscript{2+} concentration and their apparent reduction in numbers in the lamina propria in abnormally low Ca\textsuperscript{2+} concentration.

The development of endophytic growth and its suppression by high Ca\textsuperscript{2+} is most probably related to the involvement of Ca\textsuperscript{2+} in maintaining cellular cohesiveness. Most cellular Ca\textsuperscript{2+} is located on the cell surface or in the intercellular spaces, and has been shown to be very loosely bound (11). In the presence of 0.3 Ca\textsuperscript{2+} in the medium, surface-bound Ca\textsuperscript{2+}, which is at a concentration of about 1 mm (15), would be expected to be lost by simple gradient diffusion (11). This loss of Ca\textsuperscript{2+} would result in lowered cohesive forces between epithelial cells. It is possible that the loss of cohesiveness creates a distortion in the normal direction of the maturation process of the epithelium, such that cells are allowed to grow down into the stroma. Growth per se does not appear to be a causative factor in the development of endophytic growth, since conditions were found in which excess growth occurred without endophytic growth. The stroma may also play an important role in blocking endo-
phytic growth, since, as discussed above, fibroblasts appeared to be stimulated by high Ca\(^{2+}\) concentration. In the presence of high Ca\(^{2+}\) concentration, stromal elements may thus provide resistance to the downward growth of the epithelium.

When the Ca\(^{2+}\) concentration was decreased to 0.075 mM and below, there was considerable loss of cohesiveness between epithelial cells, which resulted in cells dissociating from the main epithelial mass and infiltrating into the lamina propria. The presence of epithelial cells in the lamina propria indicates a breakdown in the basement membrane. This observation combined with the evidence of lytic activity in the lamina propria suggests that epithelial cells and/or fibroblasts may have elaborated proteolytic enzymes. These enzymes could come from degenerating cells or living cells, since it has been shown that the removal of tissue Ca\(^{2+}\) stimulates the leakage of proteins from otherwise intact cells by increasing cell permeability (12).

Although this study deals only with the effect of Ca\(^{2+}\) in vitro, it is likely that Ca\(^{2+}\) is also a major factor in the control of growth in the bladder epithelium in vivo. This contention is supported by in vivo observations which show that, under conditions of vitamin A deficiency, the bladder epithelium undergoes extensive hyperplasia with endophytic growth (21) and the urinary excretion of Ca\(^{2+}\) is drastically reduced (22).

There have been numerous reports in the literature showing that malignant cells have reduced affinity for each other and contain lower amounts of Ca\(^{2+}\) than normal cells (4, 6, 7, 9). In light of these earlier studies, our present study, which shows a close similarity between the pattern of epithelial growth induced in vitro by low Ca\(^{2+}\) concentration and the growth pattern that develops in vivo during the preneoplastic phase of experimental bladder cancer, strongly suggests that there may be an early change in some aspect of cellular Ca\(^{2+}\) (probably decreased binding) in epithelia and/or stroma that have been exposed to bladder carcinogens. Furthermore, the ability to stimulate infiltrative growth by abnormally low Ca\(^{2+}\) concentration in the medium also suggests the possibility that invading bladder cancer cells may have defects in control mechanisms which are normally regulated by Ca\(^{2+}\).

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REFERENCES

All tissues were fixed in neutral formalin, embedded in glycol methacrylate, sectioned at 2 μm, and stained with toluidine blue.

Fig. 1. A, a half-bladder explant cultured for 14 days in MEM-EBS; B, other half of same bladder cultured 14 days in MEM with Ham's F12 salts. × 20.

Fig. 2. Half-bladder explants, from same bladder, cultured for 14 days in MEM with F12 salts. A, 0.3 mM Ca²⁺; B, 1.8 mM Ca²⁺. × 20.

Fig. 3. Higher power view of Fig. 2 showing difference in cellular organization. A, 0.3 mM Ca²⁺; note dysplasia in flat region of epithelium at left. Arrows, cells lining epithelial-stromal border. B, 1.8 mM Ca²⁺; note ordered transitional morphology. × 220.
Fig. 4. Half-bladder explants, from same bladder, cultured for 14 days in MEM with F12 salts. A, 1.8 mM Ca\(^{2+}\); note separation of epithelium from stroma and flattened cells (indicated by arrows) that were lining the epithelial-stromal border. B, 0.3 mM Ca\(^{2+}\); note absence of clearly defined epithelial-stromal border. × 330.

Fig. 5. A, a half-bladder explant cultured for 14 days in Ham's F12 medium; B, other half of same bladder cultured for 14 days in Ham's F12M medium; note extensive hyperplasia but absence of endophytic growth. × 20.
Fig. 6. Explant cultured for 14 days in 0.075 mM Ca++. Medium was MEM-EBS containing 11 mM glucose and 2 mM sodium pyruvate. x 330.

Fig. 7. Explant cultured for 14 days in 0.025 mM Ca++. Details same as for Fig. 6. x 330.

Fig. 8. Explant cultured for 14 days in 0.075 mM Ca++. Details same as for Fig. 6. x 540.
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