Isolation of rat mammary epithelial cells for polyamine analysis

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Mammary epithelial cells were isolated from either abdominal–inguinal glands or mammary tumours of rats, after enzymic digestion of the tissues, and were analysed for polyamine content. Optimum conditions were developed for the isolation of cells in sufficient yield for the analysis of polyamines from 1 g of mammary gland or 0.5 g of tumour tissue. Complete recoveries of the polyamines in the tissues were achieved in the isolated epithelial cells.

The aliphatic polyamines spermidine and spermine and the diamine putrescine are present in all cells of higher eukaryotes (Pegg & McCann, 1982). These compounds have a fundamental role in the diverse cellular processes of growth and differentiation (Heby, 1981). Early evidence of the essential requirement for polyamines in the growth of prokaryotic cells (Herbst & Snell, 1948, 1949) has been reinforced by the extensive documentation of the role of polyamines in growth processes, both normal and malignant, of eukaryotic cells and tissues (Jänne *et al.*, 1978). Accumulation of polyamines has been observed to accompany rapid cell growth and proliferation (Dykstra & Herbst, 1965; Russell, 1973), and transformed cells have higher concentrations of polyamines as compared with normal cells of the same origin (Russell, 1973; Bachrach, 1976; Jänne *et al.*, 1978).

Our laboratories have been studying changes in polyamine metabolism during rodent mammary-gland carcinogenesis. The induction of mammary cancer in female Sprague–Dawley rats by a single intravenous or subcutaneous dose of MNU provides a useful model for the experimental study of breast cancer (McCormick *et al.*, 1981; Thompson & Meeker, 1983). The mammary gland of the rat contains two major types of cells, epithelial (secretory and myoepithelial) and adipose, the proportions of which vary widely depending on the developmental stage of the gland (Topper *et al.*, 1975). The resting mammary gland is predominantly fat and connective tissue, with relatively few cells of epithelial origin (Russell & McVicker,

1972). During pregnancy, there is a large increase in the epithelial-cell component of the gland, which is maintained during lactation and thereafter regresses to the pre-pregnancy state. In order to study the metabolic changes in a particular cell type in response to a stimulus, it is therefore highly desirable to work with a homogeneous population of that cell type.

The isolation of epithelial cells from the mammary gland of virgin animals in sufficient quantities for biochemical studies poses considerable difficulties because of the paucity of epithelial cells relative to fat and connective tissue at this developmental stage of the gland and the relative resistance of the stromal component of the gland to enzymic digestion. In a number of studies whole-mammary-gland homogenates have been used and the resulting biochemical data reflect only the composite of changes in heterogeneous populations of cells of parenchymal, connective and adipose origin (Russell & McVicker, 1972; Brosnan *et al.*, 1982, 1983). Workers in several laboratories have isolated epithelial cells of mammary tissue after collagenase digestion (Moon *et al.*, 1969) or digestion with collagenase plus trypsin (Minasian & Jabara, 1974). In preliminary experiments we found these procedures to be unsuitable for the quantification of changes in the polyamine content of epithelial cells during mammary-gland carcinogenesis, since significant leakage of polyamines from the cells occurred during the isolation procedures. The studies reported here were undertaken to develop an improved method for the isolation of epithelial cells from the mammary gland of virgin rats that would decrease the losses of cellular polyamines.

Abbreviations used: MNU, 1-methyl-1-nitrosourea; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Materials and methods

Chemicals

Collagenase was purchased from Boehringer (Mannheim, Germany), and trypsin (type III) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). MNU was purchased from Ash Stevens Co. (Detroit, MI, U.S.A.). Medium 199 was purchased as a 10-fold concentrate (with modified Earle's salts and L-glutamine, but without NaHCO_3) from Gibco Laboratories (Grand Island, NY, U.S.A.).

Animals

Virgin female Sprague-Dawley rats were obtained from Taconic Farms, Germantown, NY, U.S.A. The animals were housed (four rats per cage) in a controlled environment at 25°C and 50% relative humidity with a 12h-light/12h-dark cycle. Distilled water and AIN-76 diet were provided *ad libitum*. Rats from which mammary tumour tissue was obtained were killed 8 weeks after a subcutaneous injection of the animals at 50 days of age with MNU (50mg/kg body wt.), dissolved in 0.9% NaCl acidified with acetic acid to pH 4.0 as previously described (Thompson & Meeker, 1983).

Epithelial-cell isolation

The rats were killed by inhalation of CO_2 . Tumours or abdominal-inguinal mammary glands were excised rapidly after removing lymph nodes. Thereafter all procedures were carried out on ice unless otherwise noted. To minimize error owing to the variation in polyamine content of different glands and tumours, tissue from several rats was pooled. Excised tissue was minced thoroughly with iris scissors for 4 min and subdivided for comparative studies. Four replicates were used for each isolation technique.

Collagenase digestion

Epithelial cells were isolated from mammary glands by modifications of the procedure described by Moon *et al.* (1969). A 1g sample of tissue from pooled and minced glands was added to 2ml of 0.25M-sucrose and 8ml of medium 199, pH 7.0, containing 10mg of collagenase, and incubated for 90 min at 37°C with vigorous shaking. The digest was vacuum-filtered through two layers of no. 109 nylon mesh. Mammary epithelial cells were separated from fat-cells by centrifugation at 400g for 10 min at 4°C. The pellet was washed twice with medium 199 and the cells were checked for viability by Trypan Blue dye exclusion. For the isolation of cells from mammary tumours, 20mg of collagenase/g of tumour tissue was used for the digestion.

Digestion with collagenase and trypsin

Modifications of the procedure of Minasian & Jabara (1974) were employed for the isolation of mammary-gland and mammary-tumour epithelial cells. A 1g sample of mammary gland, or 0.5g of tumour, was added to 5ml of medium 199, pH 7.4, containing 50mg of collagenase and 0.1ml of 12.5% (w/v) trypsin and incubated for 20 min at 37°C with vigorous shaking. The remaining procedures of filtration, centrifugation and washing of the cell pellets were identical with those used in the digestion with collagenase described above.

Polyamine and DNA analysis

The washed cell pellets obtained after the enzymic digestion and epithelial-cell isolation procedures were suspended in 1 ml of cold 5% (v/v) sulphasalicylic acid and treated for 10 s with the Brinkman Polytron sonicator. The resulting homogenate was left on ice for 1 h, centrifuged at 1000g for 5 min at 4°C, and the supernatant was decanted and utilized for polyamine analysis. The precipitate collected by centrifugation was used for DNA analysis.

The analysis of polyamines was performed by the modification of the Seiler & Wiechmann (1967) fluorescence-scanning method for dansyl derivatives of polyamines on t.l.c. plates previously described (Herbst & Dion, 1970). Toluene was substituted for benzene for the extraction of the dansyl derivatives of the polyamines, and toluene extracts were applied to t.l.c. plates with a Gilson Pipetman. The area of dansyl-polyamine fluorescence on the t.l.c. plates was quantified with a Shimadzu model CS-920 high-speed t.l.c. scanner.

The precipitates collected by centrifugation of homogenates of tumour tissue or isolated epithelial cells in 5% sulphasalicylic acid were analysed for DNA by the procedure of Burton (1956).

Results and discussion

Although the mammary glands of virgin rats are composed of predominantly stromal cells and relatively few epithelial cells, the latter are considered the cell type from which mammary-gland carcinomas arise (Russo *et al.*, 1983). It is therefore imperative that biochemical changes in epithelial cells, rather than tissue homogenates, of normal mammary tissue and tumour tissue be compared. The major aim of the present study was to obtain intact mammary-gland epithelial cells for the analysis of polyamines by procedures in which losses of these compounds were minimized. Mammary-tumour tissue homogenates, a source of predominantly epithelial cells, were utilized as a standard on which recoveries of polyamines in

epithelial cells isolated from the tumour tissue could be evaluated.

Direct analysis of polyamines on homogenates of individual mammary tumours from the same rat revealed substantial differences in the polyamine contents of the tumours (Table 1). These results suggested the necessity of the preparation of a pool of tissue for homogenates and for the preparation of epithelial cells. Minces of pooled mammary tumours or normal abdominal-inguinal mammary glands, prepared as described in the Materials and methods section, were utilized in all subsequent experiments in the present study.

The original collagenase-digestion procedure of Moon *et al.* (1969) for the isolation of the epithelial cells produced low yields of cells severely contaminated with connective tissue. The procedure was improved, with respect to the yield of epithelial cells free of connective-tissue contamination, by mincing tissues in 0.25M-sucrose, decreasing the concentration of collagenase (10mg/g of tissue) and filtering the enzymic digest through two layers of no. 109 nylon mesh. Although the recoveries of spermine and putrescine in the cells isolated by the modified procedure were satisfactory, severe losses of spermidine occurred (Table 2). Other modifications of the conditions of

digestion, concentration of collagenase, and washing and filtration procedures did not prevent the losses of spermidine.

As an alternative to the collagenase procedure, the collagenase-and-trypsin digestion method of Minasian & Jabara (1974) was evaluated. Several modifications, including changes in the concentration of the enzymes, conditions of incubation, amount of tissue, and volume of the digestion mixture, were assessed. The optimum conditions selected included the incubation of 0.5g of tumour tissue in 5ml of medium 199, pH 7.4, containing 50mg of collagenase and 2.5mg of trypsin for 20 min at 37°C. A second 6 min period of incubation described in the original procedure was found to be unnecessary and was eliminated. Furthermore, the isolated cell pellet was washed with medium 199 instead of 0.25M-sucrose. Satisfactory yields of intact viable cells (>90%) of the order of 12–16% of the original 1g of tumour tissue were obtained. The recovery of the polyamines in the cells isolated by this modified procedure was excellent (Table 2). The concentration of each of the polyamines in the isolated cells was comparable with that in the pooled tumour-tissue homogenates. Slightly higher recoveries of spermine and spermidine, in epithelial cells isolated after collagenase-plus-trypsin digestion as compared with tumour tissue homogenates, may have resulted from the improved resolution on t.l.c. plates of the dansyl derivatives of the polyamines in the extracts of isolated cells.

The modified collagenase or collagenase-and-trypsin procedures were also applied to pooled samples of normal mammary tissue. A further modification of the collagenase-and-trypsin procedure applied to tumour tissue was necessary for the isolation of epithelial cells from normal mammary gland of virgin animals. Highest polyamine concentrations were obtained in cells from preparations containing 1g of mammary tissue/5ml of digestion mixture; amounts of collagenase (50mg) and trypsin (2.5mg) were unchanged. Yields of intact viable cells were of the order of 6–8% of the original 1g of mammary-gland tissue. In Table 3

Table 1. Polyamine contents of multiple mammary tumours from the same rat

From a rat with multiple mammary tumours, four tumours were excised and a 20% (w/v) homogenate of each tumour was prepared in 5% (v/v) sulphosalicylic acid. Polyamines were analysed on the supernatants and DNA was determined on the precipitates obtained after centrifugation of the homogenates by the procedures described in the Materials and methods section,

Tumour	Content (nmol/mg of DNA)		
	Spermine	Spermidine	Putrescine
1	254.0	301.9	—
2	300.9	340.5	12.88
3	389.7	303.5	7.09
4	282.3	260.6	12.18

Table 2. Polyamine contents of homogenates and isolated epithelial cells of mammary tumour tissue

Several mammary tumours were utilized to prepare a pool of finely minced tissue. From the minced tumour tissue, four replicate samples were removed for the preparation of homogenates and for the isolation of epithelial cells by the two procedures. Polyamines and DNA were analysed in the homogenates or isolated cells by the procedures described in the Materials and methods section. Each value is the mean of four replicate analyses ($\pm$ S.E.M.).

Procedure	Content (nmol/mg of DNA)		
	Spermine	Spermidine	Putrescine
Direct homogenization	229.0 $\pm$ 20.2	206.2 $\pm$ 28.6	22.1 $\pm$ 1.5
Collagenase + trypsin	264.5 $\pm$ 3.1	240.0 $\pm$ 12.3	22.5 $\pm$ 2.2
Collagenase	226.3 $\pm$ 28.6	100.4 $\pm$ 12.2	19.6 $\pm$ 4.0

Table 3. *Comparison of the polyamine contents of epithelial cells isolated from normal mammary tissue by different enzymic procedures*

The mammary glands from several rats were pooled and minced finely. Replicate samples of the minced pool of tissue were utilized for the isolation of epithelial cells by the two procedures of enzymic digestion. The polyamine and DNA content of the isolated cell pellets were determined as described in the Materials and methods section. The values for the collagenase-and-trypsin procedure are the means for four replicate analyses ($\pm$ S.E.M.); for the collagenase-alone procedure, the values are the means for three replicate analyses ($\pm$ S.E.M.). Putrescine was not detected in these cells.

Procedure	Content (nmol/mg of DNA)	
	Spermine	Spermidine
Collagenase + trypsin	113.1 $\pm$ 14.0	173.8 $\pm$ 12.4
Collagenase	28.9 $\pm$ 3.1	44.5 $\pm$ 5.5

the results of polyamine analyses on cells isolated from normal tissue by the collagenase or collagenase-and-trypsin procedures are compared. The concentrations of spermine and spermidine are substantially higher in cells isolated by the modified collagenase-plus-trypsin procedure. Putrescine could not be detected in epithelial cells isolated from normal mammary tissue by either procedure. Thus the normal mammary tissue contains less diamine than does tumour tissue, in which putrescine occurred at one-tenth of the concentration of either spermine or spermidine. The recovery of spermine and spermidine in the epithelial cells isolated by the collagenase-plus-trypsin procedure cannot be evaluated by comparison with the polyamine content of a homogenate because of the mixed cell population of the normal mammary tissue (Kraehenbuhl, 1977). It can be assumed, however, on the basis of the results with tumour tissue (Table 2), that the trypsin-plus-collagenase procedure does not lead to a loss of polyamines.

The additional change in this procedure for normal tissue as compared with tumour tissue, i.e. 1g of pooled normal tissues as against 0.5g of pooled tumour tissue, without comparable increases in the enzyme concentration, is a cost-effective modification given the expense of collagenase.

The importance of studying responses to biological stimuli in a homogeneous cell population as against a mixed cell population has been emphasized by Kraehenbuhl (1977). If a biological stimulus is applied to the mammary gland, one cannot directly ascertain whether the response(s) shown by the gland can be ascribed to the component epithelial cells or the fat-cells. It

becomes essential to have a homogeneous cell population that can provide a means to analyse and characterize responses to known biological stimuli of one cell type. By using the procedure described above, it is now possible to study changes in tissue polyamine accumulation in mammary glands of virgin rats less than 50 days of age. Furthermore, the procedure permits the quantification of polyamines in cells isolated from the abdominal-inguinal glands of a single rat. Although the cells isolated by collagenase digestion alone are more viable and preferable for the initiation of mammary epithelial cells in primary culture (H. J. Thompson, unpublished work), the efficiency of combined trypsin-and-collagenase digestion with the shorter time of incubation eliminates the leakage of polyamines from these cells. This procedure will now permit elucidation of changes in polyamine metabolism that occur at early stages in the carcinogenic process chemically induced in the rat mammary gland.

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