SHORT COMMUNICATIONS

A Modified Technique for Isolating Epithelial Cell Nuclei from Rat Mammary Gland

A modification of the technique of Minasian and Jabara (1) is described for obtaining a higher yield of virgin rat mammary epithelial cell nuclei of greater purity. Modifications include the introduction of 0.32 M sucrose into the mammary epithelial cell-homogenizing medium and the addition of 0.05% Triton X-100 for 30 min to the crude nuclear pellet. The isolated nuclei were judged to be of acceptable purity by the virtual absence of specific cytoplasmic enzyme activities in biochemical assays and in the DNA, RNA, and protein contents of the preparations. However, electron microscopy revealed some residual contamination by cytoplasmic fragments and substantial numbers of mast-cell granules.

Minasian and Jabara (1) described a technique for isolating pure viable epithelial cells and their nuclei from virgin rat mammary glands, in sufficient quantity for biochemical studies. The criterion they adopted for nuclear purity was based on the fact that little or no contamination of the nuclei by cytoplasmic fragments was apparent by light microscopy. However, electron microscopic examinations of light microscopically 'pure' nuclei from various tissues have often been reported to show marked cytoplasmic contamination (2). Hence, interpretation of nuclear purity, based only on light microscopy, may be misleading. Such a suspicion was confirmed in relation to nuclei derived by the method of Minasian and Jabara (1) when it was found that the level of RNA in the nuclei appeared to be too high with respect to the RNA values in the cytoplasm and the whole cell.

This paper describes a modification of the Minasian and Jabara (1) procedure for isolating virgin rat mammary epithelial cell nuclei which biochemically appear to be virtually free of cytoplasmic contamination.

MATERIALS AND METHODS

Preparation and dissociation of virgin rat mammary tissue and isolation of the mammary epithelial cells have been previously reported (1).

Modified method for isolating virgin normal rat mammary gland epithelial cell nuclei. The pooled epithelial cell pellet was suspended in 2 ml of 0.32 M sucrose–3 mM MgCl₂–4 mM K₂HPO₄–4 mM KH₂PO₄ (homogenizing medium) and was homogenized in a Teflon-glass Potter–
Elvehjem type homogenizer (pestle clearance 0.006–0.01 in). Homogenization was complete after 80 strokes, with a pestle speed of 600 rpm. The homogenization was performed in an ice bath to avoid heating effects. Centrifugation of the homogenate at 700g for 15 min separated the crude nuclear fraction from the supernatant (cytoplasm). The crude nuclear pellet was washed once in 2 ml of homogenizing medium and was then suspended in 0.05% final concentration of Triton X-100 detergent (Rohm and Haas Aust. Pty. Ltd., Melbourne, Australia), placed in an ice bath for 15 min, and then centrifuged at 700g for 15 min; the supernatant was discarded. The pellet was resuspended in 2 ml of homogenizing medium, made up to a final concentration of 2 M sucrose–3 mM MgCl₂. The suspension was centrifuged for 1 hr at 50,000g in the Type 65 rotor of the Spinco Model L preparative ultracentrifuge. The nuclei sedimented to the lower bottom side of the tube, and contaminating material was observed at the top of the tube.

Nuclear purity. Nuclear purity was determined (a) biochemically, by assaying for the following specific cytoplasmic enzymes in the nuclear fraction: lactic dehydrogenase (3), pyruvate kinase (4), and succinate dehydrogenase (5); and (b) by electron microscopic examination of the nuclear fraction following its fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, postfixation in 1% osmium tetroxide and embedding in araldite. Ultrathin sections stained with uranyl acetate and lead nitrate were examined in a Philips EM 300 electron microscope.

DNA, RNA, and protein determinations. DNA, RNA, and protein were isolated by the method of Munro and Fleck (6). Nuclear, cytoplasmic, and whole-cell DNA was estimated by the indole method of Ceriotti (7), RNA by absorbance at 260 nm, and nuclear protein by the method of Lowry et al. (8).

RESULTS AND DISCUSSION

Modifications Made to Nuclear Isolation Technique

The modifications introduced into Group 2b (Table 1) proved to be the most effective in reducing nuclear cytoplasmic contamination and have now been adopted as standard procedure for isolating rat mammary nuclei in this laboratory. Introduction of 0.32 M sucrose to the homogenizing medium, as suggested by Tata (2) for the isolation of liver cell nuclei, resulted in a more compact pellet in contrast to the loose one produced by the original method and, in addition, reduced the nuclear RNA:nuclear protein ratio (Table 1). Inclusion of 0.05% Triton X-100 for 30 min resulted in a further reduction in the nuclear RNA:nuclear protein ratio, indicating a greater decrease in nuclear cytoplasmic contamination (Table 1). Triton X-100 is a mild nonionic detergent which separates double plasma membranes and, therefore, probably removes
TABLE 1

COMPARISONS OF RNA AND PROTEIN FROM RAT MAMMARY EPITHELIAL NUCLEI WHICH HAVE BEEN PURIFIED BY THE ORIGINAL METHOD OR ITS MODIFICATIONS

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment applied to nuclear fraction</th>
<th>Nuclear protein (μg)</th>
<th>Nuclear RNA: nuclear protein (μg/mg)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (a)</td>
<td>Original homogenizing buffer: 0.0074 M Na₂HPO₄-0.006 M KH₂PO₄ (pH 7.4)-0.075 M NaCl (I)</td>
<td>1800</td>
<td>132</td>
<td></td>
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<tr>
<td></td>
<td>Homogenizing buffer: 0.32 M sucrose-3 mM MgCl₂-4 mM KH₂PO₄, 4 mM K₂HPO₄ (pH 7.4) (II)</td>
<td>1800</td>
<td>78</td>
<td>-41</td>
</tr>
<tr>
<td>2 (a)</td>
<td>II (b) II plus 0.05% Triton X-100 (30 min) (III)</td>
<td>2400</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>3 (a)</td>
<td>III (b) II plus 0.09% Triton X-100 (30 min)</td>
<td>2040</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>4 (a)</td>
<td>III (b) II plus 0.05% Triton X-100 (45 min)</td>
<td>2050</td>
<td>68</td>
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For each main group, equal numbers of rats from the same litter were randomly allocated to a control (a) or a modified subgroup (b). Therefore, within-group comparisons could be made, but not comparisons between the main groups.

Attached ribosomes which may be adhering to the outer nuclear membrane; it has been used for purifying isolated nuclei derived from other tissues (2,9).

Biochemical Determinations of Nuclear Purity

Assays for lactic dehydrogenase and pyruvate kinase both revealed marked reductions in nuclear contamination by cell organelles other than mitochondria using the modified method, compared with the original technique (Table 2). Succinate dehydrogenase assay, which was used as a specific mitochondrial enzyme marker, suggested that mitochondrial contamination was minimal with either method (Table 2). Two possible explanations for this result are either that both methods are equally effective in eliminating mitochondria from the nuclear preparation, or, more likely, virgin rat mammary epithelial cells probably contain very
TABLE 2
ASSAYS FOR SPECIFIC CYTOPLASMIC ENZYMES IN MAMMARY EPITHELIAL CELL
SUBCELLULAR FRACTIONS DERIVED BY THE ORIGINAL OR
MODIFIED ISOLATION METHOD

<table>
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<th>Assay</th>
<th>Original method</th>
<th>Modified method</th>
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<tr>
<td></td>
<td>Nuclear EA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cytoplasmic EA &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactic dehydrogenase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.5 ± 0.71&lt;sup&gt;c&lt;/sup&gt;</td>
<td>73.5 ± 0.71</td>
</tr>
<tr>
<td>Pyruvate kinase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20.5 ± 0.71</td>
<td>78.5 ± 0.71</td>
</tr>
<tr>
<td>Succinate dehydrogenase&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
<td>100</td>
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</table>

<sup>a</sup> EA, enzyme activity.
<sup>b</sup> Lactic dehydrogenase activity expressed in Wrobleski unit/ml of suspension.
<sup>c</sup> Each value represents the mean percentage ± standard deviation of three separate determinations each of six rats, using 12 glands per rat from the thoracic-abdomino-inguinal regions.
<sup>d</sup> Pyruvate kinase activity expressed as the OD change/min as 1 μmol of pyruvate is produced/min.
<sup>e</sup> Succinate dehydrogenase activity measured as the OD change/3 min.

Few mitochondria. A search of the literature failed to find a report on the ultrastructure of the mammary gland of the virgin rat, but, in the virgin mouse mammary gland, electron microscopy has revealed only a few small sparsely scattered mitochondria in the epithelial cells; mitochondria increased markedly in both size and number only during pregnancy and lactation (10).

Fig. 1. Example of a nucleus from which cytoplasm has been removed. (×13,400).
Electron microscopic examination of the nuclear fraction produced by the modified method revealed many nuclei essentially free of cytoplasmic contamination (Fig. 1). Cytoplasmic remnants were, however, present in these preparations, both attached to the surfaces of some nuclei and apparently free (Fig. 2). Membranous elements were always seen within these remnants, but identifiable cytoplasmic organelles were seldom present, supporting the biochemical findings. Only occasionally, cells were seen which appeared largely intact. In addition, preparations contained a few small aggregates of collagen microfibrils and large numbers of structures closely resembling mast-cell granules (Fig. 3). These granules, which were membrane-bound, were round or oval in outline, ≈0.9 μm in diameter (range 0.6–1.3 μm), and were composed internally of a moderately electron-dense finely granular material. Further evidence of their identity as mast-cell granules was provided by positive metachromasia in toluidine blue-stained preparations of fresh material. The abundance of these granules is, perhaps, not surprising in view of the large numbers of mast cells to be seen in paraffin sec-

**Fig. 2.** Nuclei (N) associated with some remnants of cytoplasm (C), containing membranous elements but lacking recognizable organelles. A few presumed mast-cell granules (MG) are present. (×9800).
Fig. 3. A small cluster of presumed mast-cell granules (MG). Part of an isolated nucleus (N) is also present. (×9200).

ations of virgin rat mammary glands (unpublished data). Introduction of a final step-gradient ultracentrifugation of 2.1–2.5 M sucrose in 0.1 M steps, with 3 mM MgCl₂, failed to separate the granules from the nuclei, suggesting that the two are of approximately the same density. However, the granules neither interfere with the present biochemical assays nor affect the biochemical results.

DNA, RNA, and Protein Contents

Table 3 presents the results from four separate determinations of normal rat mammary epithelial cell and subcellular protein, DNA, and RNA contents, using the modified nuclear isolation method. In contrast to the original technique (1), the yield of pure nuclei to crude nuclei was markedly increased. However, the RNA/DNA ratio remained approximately the same with either method. This finding, together with the results of the biochemical assays, suggests that nuclei obtained by the

| TABLE 3 | DETERMINATIONS OF RAT MAMMARY EPITHELIAL CELL AND SUBCELLULAR PROTEIN AND NUCLEIC ACID CONTENTS

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<thead>
<tr>
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<th>Whole cell</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
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<tr>
<td>Protein (µg): cell pellet (mg wet wt)</td>
<td>4.03 ± 0.23</td>
<td></td>
<td></td>
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<tr>
<td>RNA (µg): nuclear protein (mg)</td>
<td>142 ± 18</td>
<td>69 ± 1.40</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>DNA (µg): nuclear protein (mg)</td>
<td>376 ± 8.47</td>
<td>329.98 ± 16.50</td>
<td>22 ± 6.48</td>
</tr>
<tr>
<td>RNA (µg): DNA (µg)</td>
<td>0.21 ± 0.01</td>
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Values represent the means ± standard deviations of four separate determinations each of six rats, using 12 glands per rat from the thoracic–abdomino–inguinal regions. Calculations were based on the following mean wet weights ± SD: total gland = 17.21 ± 5.02 g; cell pellet: gland = 9 ± 3%; crude nuclear pellet = 0.47 ± 0.06 g; pure nuclear: crude nuclear pellet = 66 ± 3%.
original method, although showing considerable cytoplasmic contamination, were not contaminated by organelles rich in RNA.

This modified method for the isolation of virgin rat mammary epithelial cell nuclei is simple, rapid, reproducible, and suitable for studies in physiology, endocrinology, and experimental carcinogenesis.

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REFERENCES


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