Rapid Method for Preparation of Nuclei from a Mucin-producing Solid Tumor

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ABSTRACT

Nuclei were prepared from the human colon mucin-producing tumor GW-39. The method described involves incubation in β-mercaptoethanol under mild conditions and results in the preparation of nuclei free of the mucin web that initially enmeshes them. A sensitive colorimetric immunoperoxidase assay was used to confirm that nuclei prepared in this fashion were generally free of contaminating mucin. This method is mild, rapid, and should be applicable to other mucin-producing tumors.

INTRODUCTION

The preparation of nuclei from mammalian cells is most commonly based on the principle of differential centrifugation in buffered hypertonic and isotonic solutions (12, 23). When this approach is applied to tumors, either freshly excised or propagated in laboratory animals, the situation is confounded by the heterogeneity of cell types (2, 7, 23), the presence of necrotic cells, and in some cases production of mucin (30). The production of mucin is particularly troublesome because it not only is a potential nuclear contaminant (20) but also, as a result of its gelatinous properties, makes nuclear separation and purification based on differential centrifugation very difficult. The viscosity, and hence potential contamination, of mucin can be decreased by the application of N-acetylcysteine (19) or by digestion with β-glucuronidase or neuraminidase (30). More drastic conditions, such as the use of proteolytic agents (1, 24) or the phenol:water extraction of mucin (14), are generally considered too harsh to maintain the morphological integrity of nuclei and chromatin.

The present paper describes a mild treatment with β-mercaptoethanol to disrupt the mucin gel and allow for the preparation of purified nuclei by traditional means. A sensitive peroxidase:antiperoxidase assay has been adapted to demonstrate that the nuclear preparation is generally free of contaminating mucin, known to be produced in large amounts by this tumor (30). This method should be applicable to other mucin-producing tumors, is milder than enzymatic digestions, and serves as a rapid and convenient method of preparing nuclei from these tumors.

MATERIALS AND METHODS

Propagation of Tumor. The mucin-producing human colon adenocarcinoma cell line GW-39 was a gift from Dr. David Goldenberg (University of Kentucky Medical Center, Lexington, Ky.) and was propagated as a serially transplanted solid tumor in the cheek pouch of unconditioned Syrian hamsters. Tumors were grown in the hind leg muscle for harvesting. Growth in cheek pouches or leg muscle was from a fine minceate of tumor cells in sterile Hank’s balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) containing penicillin and streptomycin.

Preparation of Nuclei. All solutions used for the preparation of nuclei contained 0.1 mM phenylmethylsulfonyl fluoride and were used at 0–4°. All homogenizations were done with a loose-fitting Teflon pestle unless stated otherwise. Connective tissue and muscle were trimmed, and excess blood was removed by rinsing the tumor in 0.25 M sucrose:0.01 M Tris, pH 7.5. The tumor was minced finely and homogenized in 0.2 M β-mercaptoethanol:5 mM MgCl₂:0.15 M NaCl:0.1 M Tris, pH 8.5, using a tightly fitting Teflon pestle to reduce mucin viscosity (1). The homogenate was stirred for 30 min on ice and then centrifuged at 3000 × g for 10 min. The pellet (Pellet 1) was homogenized in 0.1 M β-mercaptoethanol:5 mM MgCl₂:0.25 M sucrose:0.01 M Tris, pH 8.5, and filtered through 4 layers of gauze. The suspension was centrifuged at 900 × g for 10 min, and the resulting pellet (Pellet 2) was resuspended in 0.25 M sucrose:0.01 M Tris, pH 7.5. The preparation of nuclei and chromatin from this material was as described by Spelsberg et al. (25). Briefly, the crude nuclei were collected by centrifugation at 900 × g for 10 min, and the resulting pellet (Pellet 3) was homogenized in 2.2 M sucrose:5 mM MgCl₂:0.01 M Tris, pH 7.5. The homogenate was underlaid with additional 2.2 M sucrose:5 mM MgCl₂:0.01 M Tris, pH 7.5, and centrifuged at 93,000 × g for 1 hr. Nuclei (Pellet 4) were suspended in 0.25 M sucrose:0.5% (w/v) Triton X-100:0.01 M Tris, pH 7.5, and collected as a pellet (Pellet 5) after centrifugation at 900 × g for 10 min. A summary of the nuclear preparation may be found in Chart 1.

The material in Pellet 5 was fixed at 4° in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). After 2 hr, the pellets were rinsed extensively in the buffer and postfixed 90 min in the same buffer containing 1.0% OsO₄. Following ethanol dehydration, samples were transferred through propylene oxide prior to embedding in Epon.

Areas for electron microscopic analysis were selected randomly throughout the pellets after light microscopic examination of 1-μm-thick sections stained with toluidine blue. Thin sections were mounted on naked copper grids, stained with uranyl acetate and lead citrate, and examined in an Hitachi Model H-600 electron microscope operated at 75 kV.

Assay for Mucin Contamination. Contamination by mucin was determined colorimetrically using the peroxidase:antiperoxidase method as described (13, 27) with ABTS as electron donor (29). Nuclei (5 ml aliquots at various stages of purification) were collected by centrifugation at 4000 × g for 7 min. Each incubation was for 1 hr at room temperature with constant agitation. The order and dilution (in 1 ml of PBS) of immunoreagents were as follows: peanut agglutinin (50 μg/ml; Sigma Chemical Co., St. Louis, Mo.); 1:40 dilution of antipeanut agglutinin made in rabbit (E-Y Laboratories, San Mateo, Calif.); 1:40 dilution of anti-rabbit IgG made in goat (Sera Resources, Inc., Berlin, Mass.); and 1:200 dilution of peroxidase:antiperoxidase made in rabbit (prepared by J. Briggs in this laboratory). After each incubation, the pellets were collected by centrifugation at 4000 × g for 7 min and washed 2 times by suspension and

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centrifugation in 4 ml of PBS (5 washes in PBS after incubation with peroxidase-antiperoxidase). The final color reaction was produced by adding ABTS (1.14 mg/ml; Sigma) and 0.005% H₂O₂ in sodium phosphate buffer (3.33 mM Na₂HPO₄:0.67 mM NaH₂PO₄, pH 6.0). The color reaction was stopped by adding 0.1 volume of 5 mM sodium azide (22), and the absorbance (at 420 nm) of the supernatants was determined.

Analytical Methods. DNA and RNA content was determined on HClO₄ precipitates by the diphenylamine and orcinol reactions, respectively, as described by Ch'ih et al. (9). Protein content was determined by the method of Lowry et al. (21) using bovine serum albumin as standard. Gel electrophoresis was as described previously (10), and molecular weights were approximated by comparing the relative mobilities of proteins to those of known molecular weights (Bio-Rad Laboratories, Richmond, Calif.). Complement fixation was according to the method of Wasserman and Levine (28) as described previously (10) using 75 pg proteins to those of known molecular weights (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

The production of large amounts of mucin by some adenocarcinomas requires the dispersion of cells and nuclei before chromatin can be prepared by classical means. In the case of GW-39 tumor, this was accomplished by incubation in the presence of β-mercaptoethanol as shown in Chart 1. Phase contrast photomicrographs of the resulting nuclear preparation are shown in Fig. 1. When the tumor minceate was homogenized and incubated in 0.2 M β-mercaptoethanol:5 mM MgCl₂:0.15 M NaCl:0.1 M Tris, pH 8.5, the resulting homogenate consisted of intact cells arranged in clusters which were enmeshed in mucin lacunae as described by Yeoman et al. (30). This gelatinous suspension also contained large amounts of debris including extranuclear material including membrane fragments (Fig. 2). The suspension was much less viscous and contained primarily cell plasmic and noncellular debris were abundant as was the highly refractile material. When the crude nuclei were collected by centrifugation and suspended in 2.2 M sucrose:5 mM MgCl₂:0.15 M NaCl, pH 7.5 (Pellet 3), the nuclei became pyknotic as a result of the hypertonic conditions. The suspension also contained extranuclear debris as well as some of the highly refractile material. The supernatant, after the 93,000 × g centrifugation, contained much particulate debris, highly refractile material, distorted and damaged nuclei, and nuclei with prominent cytologic tags (data not shown). The corresponding pellet (Pellet 4) in 0.25 M sucrose:0.5% (v/v) Triton X-100:0.01 M Tris, pH 7.5, contained intact, well-rounded nuclei which appeared translucent and free nuclei were rarely present. Pellet 2, obtained after centrifugation of the filtrate, was firmer and less gelatinous than Pellet 1 and, when suspended in 0.25 M sucrose:0.01 M Tris, pH 7.5, consisted of intact nuclei with few whole cells present. Cytoplasmic and noncellular debris were abundant as was the highly refractile material. When the crude nuclei were collected by centrifugation and suspended in 2.2 M sucrose:5 mM MgCl₂:0.01 M Tris, pH 7.5 (Pellet 3), the nuclei became pyknotic as a result of the hypertonic conditions. The suspension also contained extranuclear debris as well as some of the highly refractile material. The supernatant, after the 93,000 × g centrifugation, contained much particulate debris, highly refractile material, distorted and damaged nuclei, and nuclei with prominent cytologic tags (data not shown). The corresponding pellet (Pellet 4) in 0.25 M sucrose:0.5% (v/v) Triton X-100:0.01 M Tris, pH 7.5, contained intact, well-rounded nuclei which appeared translucent and generally free of surface debris. Very little particulate debris was present, and the highly refractile material was virtually absent. When this material was pelleted by centrifugation and resuspended in 0.25 M sucrose:0.01 M Tris, pH 7.5 (Pellet 5), the resulting nuclei appeared translucent and were fragile with some releasing their chromatin content. Examination by electron microscopy showed that the final Triton X-100 nuclear pellet was composed of nuclei devoid of both the nuclear envelope and extranuclear material including membrane fragments (Fig. 2).

Contamination by mucin was assessed by taking advantage of the specific binding of peanut agglutinin to colon tumor mucin (5, 6). The binding was detected by subsequent binding of anti-peanut agglutinin to the lectin and quantitated colorimetrically at 420 nm using the peroxidase-antiperoxidase method (10) with the soluble reagent ABTS. Chart 2 shows that the highest reactivity was found at the beginning of the nuclear preparation, and treatment of the sample with the reducing agent β-mercaptoethanol reduces the mucin content by 60%. Nuclear, after passage through 2.2 M sucrose-containing buffer (Pellet 4), show...
only 6.6% of the original $A_{420}$-absorbing material present in the whole homogenate.

The nuclear fraction was analyzed for DNA, RNA, protein content, and extranuclear contamination (Table 1). The DNA content in the whole homogenate fraction was not determined because this fraction contained sugars (glycoproteins, glycoalky) that interfere with the diphenylamine test resulting in a metachromatic reaction product that artificially elevated the absorbance. The DNA:protein ratio in the nuclear fraction (0.04) was typical of values reported for other tissues, such as rat liver (9). The DNA:RNA ratio and the negligible amount of RNA found in the nuclear preparation (0.004 mg/mg of protein) demonstrated that this nuclear isolation procedure resulted in only negligible cytoplasmic contamination. This was confirmed when contamination with an extranuclear protein, CEA, was examined by complement fixation (Table 1). While 77% of the complement was fixed in the whole homogenate fraction under these conditions, virtually no complement was fixed (and hence no CEA was present) in the nuclear fraction. The 6% complement fixed could be considered background, since antibody alone (no protein added) gave a similar value (data not shown).

When chromatin was prepared from isolated nuclei (10), a dramatic difference was observed between nuclei prepared with and without $\beta$-mercaptoethanol (Fig. 3). Major contaminating proteins of approximate molecular weights of 174,000 and 38,000 were characteristic of chromatin prepared by existing methods (10, 25) and greatly reduced or absent when prepared using $\beta$-mercaptoethanol as described in “Materials and Methods.”

**DISCUSSION**

The preparation of nuclei from the GW-39 tumor requires prior release of cells from the mucin web that enmeshes them. Procedures for preparation of nuclei outlined by Spelsberg et al. (25), successful for other cell types including colon adenocarcinoma cells grown in vitro or nonneoplastic colonic epithelial cells (10), proved to be unsuitable for the GW-39 tumor. Factors contributing to this lack of success included the viscosity and the gelatinous quality of the mucin and the enclosure of cells in the mucin sacs and lacunae characteristic of this tumor (30). As a result, the method of Spelsberg et al. (25) had to be modified to bring about release of the cells from the mucin network while minimizing mucin contamination.

Mucin is known to form large aggregates held in a 3-dimensional network by disulfide bonds (11, 24). These bonds involve not only the mucin polypeptide backbone but also contaminating proteins which are known to be entangled in the 3-dimensional mucin network (24). The viscosity of mucin can be decreased by reduction of these bonds with dithiothreitol (11) or 0.2 $\mu$ l $\beta$-mercaptoethanol (1, 24). Other work has shown that the presence of cations (1) and partial proteolysis (1, 24) also reduce viscosity. When the presence of a reducing agent was combined with 0.15 M NaCl to maintain isotonicity and 5 $\mu$ M MgCl$_2$ to maintain nuclear morphology and prevent nuclear clumping (23), the result was a less viscous suspension of individual cells which could be separated from the mucin in subsequent steps. Chart 1 outlines the procedure used. The 30-min incubation to reduce the disulfide bonds was followed by a second exposure to the reducing agent in isotonic sucrose which kept the mucin reduced while cells were pelleting through isotonic sucrose. This method is not only rapid but can be conducted at 10-40°C which retards proteolytic activity. Yeoman et al. (30) have described a successful mucin digestion procedure involving neuraminidase and $\beta$-glucuronidase, but the conditions involve a 3-hr incubation at 37°C which may have adverse effects on heat lability or phenylmethylsulfonyl fluoride-resistant proteolysis of proteins.

After the viscosity had been reduced and the cells released from the mucin network, more conventional methods for nuclear preparation could be used (25). In short, after the nuclei were washed in isotonic sucrose buffer to remove the excess $\beta$-mercaptoethanol, they were purified by centrifugation in hypertonic sucrose (8, 23). The high density of 2.2 M sucrose-containing buffer allows nuclei to pellet while keeping nonnuclear, damaged nuclear, and contaminated nuclear material in the supernatant. High viscosity also helps to remove the outer nuclear membrane by forces of shearing (4). Finally, to remove completely the outer nuclear membrane and attached cytoplasmic contaminants, the nuclei were washed in isotonic sucrose buffer containing 0.5% (v/v) Triton X-100 (4, 23). Electron microscopy confirmed that the nuclear preparation at this step was indeed lacking the outer nuclear membrane and extranuclear contaminants. The final yield of nuclear DNA was consistent with the amount reported to be present in other human tissues (3), and this value did not decrease when the material was further processed to purified chromatin by the method of Spelsberg et al. (25), suggesting that this value does indeed represent chromatin (or material that is inseparable from chromatin).

The GW-39 tumor, isolated and characterized by Goldenberg et al. (16-18), is an explant from a primary colon adenocarcinoma and has been propagated in immunocompetent hamsters since 1966. The karyotype confirms it is of human origin (17).
The tumor is known to produce large amounts of mucin, reflecting the retention of some characteristics of differentiated colonic epithelial cells (16). The mucin tends to keep the cells in clusters, which in turn are arranged in lacunae (30). Such a cluster of cells is shown in the phase contrast photomicrograph of Fig. 1A and was obtained by homogenization of the tumor mincemat. Background debris of various origins and composed of extracellular material and some cell lysate was abundant and was not removed until the nuclei were centrifuged through 2.2 M sucrose-containing buffer (Fig. 1E). The presence of Mg2+ in buffers prior to the Triton X-100 kept the nuclei intact while still allowing the cells to lyse (Fig. 1C). The highly refractile material (Fig. 1, A to D) probably represented nuclei from necrotic cells which were osmotically inactive and cosedimented with nondenatured nuclei until the 2 were separated in 2.2 M sucrose-containing buffer. Underlaying with fresh 2.2 M sucrose-containing buffer was essential for this separation, and it was very rare that the nuclear pellet contained this refractile material after this step (Fig. 1, E and F). In fact, electron microscopy revealed that the nuclear preparation was free of cosedimenting membranous material (Fig. 2). In summary, from the phase contrast photomicrographs of Fig. 1, it is clear that the mucin network was disrupted in the initial steps, allowing for the subsequent collection of nuclei in later steps.

The mucin content was indeed greatly reduced as the nuclei were increasingly purified (Chart 2). These data are based on the specific binding of peanut agglutinin to the galactosyl-1→3N-acetylgalactosamine domain of colonic tumor mucin. This reaction is specific for colon tumor mucin (5, 6), and this determinant has received recent attention as a colon tumor-specific cell surface antigen (26). The final color reaction accompanying the reduction of ABTS is probably only semiquantitative in this assay, and hence, the trend toward decreased mucin content, rather than absolute values, is of primary importance. Compared to the whole homogenate, the mucin content was reduced by 59% in Pellet 1 after treatment with β-mercaptoethanol. Further treatment with this reducing agent decreased the amount of mucin in the nuclear preparation by an additional 10% in Pellet 2. The amount of mucin in Pellet 4 was only 6.6% of that found in the whole homogenate. This 93% reduction in mucin content indicates that initial incubation in β-mercaptoethanol results in subsequent separation of nuclei from mucin.

The amount of DNA recovered in the nuclear preparation and the DNA:RNA ratio (Table 1) are characteristic of other nuclei prepared by other methods (4, 9), demonstrating that nuclei prepared in the presence of β-mercaptoethanol exhibit no adverse effects on yield or recovery (also see Fig. 2). When the extranuclear protein CEA was used as a marker protein (Table 1), it was evident that contamination of nuclei was at an absolute minimum. This was also apparent when chromatin proteins (from nuclei prepared in the presence or absence of β-mercaptoethanol) were examined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 3). It is interesting to note that the 2 contaminating proteins in chromatin from nuclei prepared in the absence of β-mercaptoethanol (but not from nuclei prepared in the presence of β-mercaptoethanol), with approximate molecular weights of 174,000 and 38,000 (Fig. 3), share the same approximate molecular weights as cell surface glycoproteins of G-39 tumors (15).

The use of β-mercaptoethanol allows for the preparation of nuclei from mucin-producing tumors in a straightforward fashion with the resulting nuclear fraction free of the mucin network and morphologically intact. This procedure should be applicable to other mucin-producing tumors and should find wide use when purified nuclei are critical for biochemical or immunological characterization.

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REFERENCES


Fig. 1. Phase-contrast photomicrographs of nuclei at various stages of preparation. Nuclei were prepared as described in Chart 1. A, whole homogenate; B, Pellet 1; C, Pellet 2; D, Pellet 3; E, Pellet 4; F, Pellet 5. x 480.
Fig. 2. Electron micrograph of Triton X-100-washed nuclei from GW-39 cells. Nuclei in Pellet 5 were fixed with glutaraldehyde and prepared for electron microscopy as described in "Materials and Methods." Bar, 1.0 μm.

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of GW-39 chromatin. Lane 1, prepared in the presence of β-mercaptoethanol; Lane 2, prepared in the absence of β-mercaptoethanol; Lane 3, molecular weight standards. Chromatin samples represent 40 μg (as DNA) applied to the gel, and molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) are: myosin, M, 200,000; β-galactosidase, M, 116,500; phosphorylase b, M, 94,000; albumin, M, 68,000; and ovalbumin, M, 45,000. Experimental details are given in "Materials and Methods."
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