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DEMONSTRATION OF CROSS-LINKED CYTOKERATIN POLYPEPTIDES IN TRANSPLANTABLE RAT HEPATOMA CELLS

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Covalently cross-linked multimers of cytokeratins were shown to be present in transplantable Morris hepatoma 7777 cells. These high molecular weight antigens were not detectable in normal rat liver cells. However, identical high molecular weight antigens were also demonstrated in rat liver cells when the cells were homogenized in solutions containing Ca^{2+}. The cross-linking reaction was suggested to be mediated by the action of tissue transglutaminases. © 1991 Academic Press, Inc.

Cytokeratins consist of a multigene family of proteins, subsets of which are differently expressed in distinct epithelial cell types (1). Rat hepatocytes contain two major cytokeratin components (55 kDa and 49 kDa) and several minor components (2), assembled as intermediate type filaments that interact with various organelles, including nuclear lamina and desmosomes (3). Changes in expression and/or organization of cytokeratins are observed in various pathological conditions (4-6).

Previously we have shown that a monoclonal antibody (MoAb) against rat cytokeratins detects high molecular weight antigens (90-120 kDa) in addition to monomeric cytokeratin polypeptides (7). Although these high molecular weight keratin-like antigens have been recognized by several investigators, their nature and pathological significance have not yet been elucidated (2,8). In this report, these high molecular weight antigens were identified as cytokeratin polypeptides cross-linked by isopeptide bonds, probably by the action of tissue transglutaminases, to each other or with other components. We have also shown that the method commonly used for liver cell fractionation may generate artifactual cross-linking of proteins during the homogenization and fractionation procedures, which seems to have been neglected by many investigators. Special attention was given to avoid such artifactual cross-linking of proteins during

<u>Abbreviations:</u> MoAb, monoclonal antibody; PBS, phosphate buffered saline; PMSF, phenylmethylsulphonyl fluoride; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulphate; PAGE, polyacryamide gel electrophoresis; TG, transglutaminase.

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preparation of samples. The results confirmed the presence of abundant cross-linked cytokeratin polypeptides in transplantable rat hepatoma cells.

Materials and Methods

Normal liver and hepatoma cells

Normal liver was removed from an adult female Buffalo rat. Transplantable Morris hepatoma 7777 was maintained in the thigh muscle of female Buffalo rats. Solid tumors were harvested when they reached 1-2 cm in diameter. McA-RH7777 cells (9), which are a subclone of transplantable Morris hepatoma 7777, were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) at 37°C in a humidified 5% CO_2 incubator. Cultured cells were harvested with a rubber policeman after the monolayer was rinsed twice with ice-cold PBS.

Homogenization and sample preparation

The following procedures were performed at 0-4°C. Small portions (0.2 to 0.3 g) of normal rat liver or tumor tissue were minced in 10 vol. of STMT buffer (0.25M sucrose/50 mM Tris-HCl, pH7.9/5 mM $MgCl_2$/0.5% Triton X-100/1 mM PMSF) with a Polytron homogenizer (Brinkmann, Westbury, NY) at top speed for 3 sec and then homogenized with a motor driven Teflon pestle in a glass homogenizer. In some experiments, one of the following reagents was added in the homogenizing solution; 5 mM $CaCl_2$, 5 mM EGTA, 10 mM iodoacetamide, or various concentrations of putrescine. The homogenates were then adjusted to 5% 2-mercaptoethanol/2% SDS/10% glycerol, sonicated for 3 sec at maximum power using a Biosonik VI (VWR scientific, San Francisco, CA), and boiled for 5 min. As controls, tissue and cells were directly homogenized and solubilized in SDS sample buffer (0.0625M Tris-HCl, pH 6.8/5% 2-mercaptoethanol/2% SDS/10% glycerol) and boiled for 5 min.

Sodium dodecyl sulfate acrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Protein samples were electrophoresed according to the system of Laemmli (10) using a 3% acrylamide stacking gel and a 9% resolving gel. Immunoblot analysis was performed as described elsewhere (11). A murine MoAb ($24A_3$), which is specific to rat liver cytokeratins, was characterized previously (12).

Results

Artifactual cross-linking of cytokeratins during homogenization in normal rat liver cells

Our previous immunoblot analysis using the MoAb $24A_3$ showed immunoreactive antigens having high molecular weights (90-120 kDa) in addition to monomeric cytokeratin polypeptides in cytoskeletal preparations from normal rat liver as well as hepatoma cells (7). Further investigation confirmed that the high molecular weight immunoreactive antigens observed in normal rat liver cells were formed artifactually during homogenization and fractionation processes (Fig. 1A, lane 2), because these antigens were not detectable in normal rat liver cells when the cells were directly dissolved in SDS sample buffer (Fig. 1A, lane 1).

10% (W/V) homogenates of normal rat liver cells were prepared in various solutions and the proteins were analyzed after incubation for 30 min at room

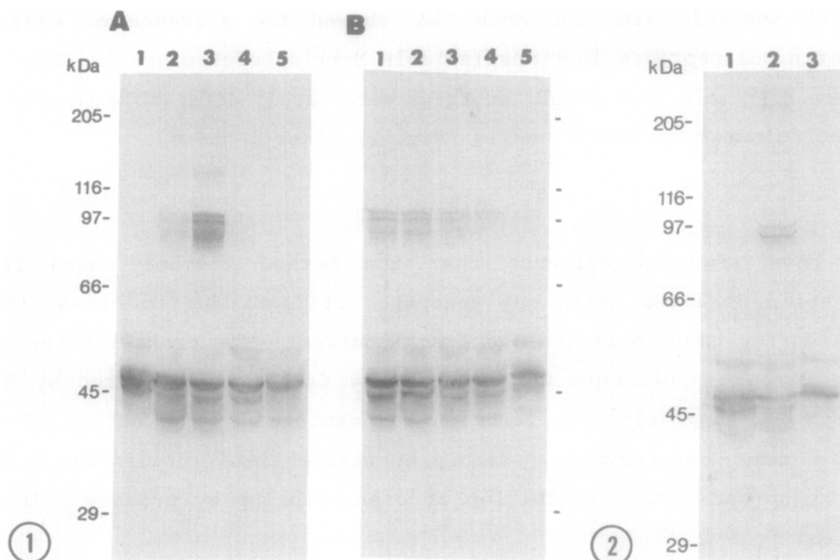

Fig. 1. Demonstration of artifactual cross-linking of cytokeratins during homogenization in normal rat liver cells. (A) Protein samples of normal rat liver cells were directly dissolved in SDS sample buffer (lane 1) or homogenized in STMT buffer alone (lane 2) or in STMT buffer containing 5 mM $CaCl_2$ (lane 3), 5 mM EGTA (lane 4), or 10 mm iodoacetamide (lane 5). After incubating for 30 min at room temperature, the homogenates were analyzed by SDS-PAGE followed by immunoblot analysis using MoAb $24A_3$. (B) Rat liver tissue was homogenized in STMT buffer (lane 1) or STMT buffer containing 0.5 mM (lane 2), 2.5 mM (lane 3), 12.5 mM (lane 4), or 62.5 (lane 5) mM putrescine and incubated for 30 min on ice. The homogenates were then further incubated for 30 min at room temperature after adding $CaCl_2$ to a final concentration of 5 mM. The immunoblot analysis using MoAb $24A_3$ was then carried out.

Fig. 2. Demonstration of cross-linked cytokeratins in transplantable Morris hepatoma 7777 cells. Normal rat liver cells (lane 1), transplantable hepatoma 7777 cells (lane 2) and in vitro cultured McA-RH 7777 cells (lane 3) were directly dissolved in SDS sample buffer and the solubilized proteins were analyzed by SDS-PAGE followed by immunoblot analysis using MoAb $24A_3$.

temperature. The appearance of high molecular weight antigens was enhanced by addition of Ca^{2+} (Fig. 1A, lane 3) and inhibited in solutions containing EGTA, or iodoacetamide. (Fig. 1A, lanes 4 and 5). Fig. 1B shows inhibition of Ca^{2+}-induced cross-linking of cytokeratins by putrescine. In this experiment, rat liver tissue was homogenized and incubated in STMT buffer containing various concentrations of putrescine for 30 min on ice, and then further incubated for 30 min at room temperature after adding $CaCl_2$ to a final concentration of 5 mM.

Similar results were also observed for another isotonic solution commonly used in the fractionation of liver tissue, such as STM (0.25M sucrose/50 mM Tris-HCl, pH 7.5/5 mM $MgCl_2$), TNM (10 mM Tris-HCl, pH 7.6/140 mM NaCl/5 mM $MgCl_2$), calcium and magnesium-free Dulbecco's phosphate buffered saline (138 mM NaCl/8.1 mM Na_2HPO_4/2.7 mM KCl/1.1 mM KH_2PO_4) (data not shown).

Presence of cross-linked cytokeratins in transplantable rat hepatoma cells.

Because of the above artifacts, tissue and cells were directly dissolved in SDS sample buffer and the patterns of cytokeratin polypeptides were analyzed.

Immunoblot analysis with the MoAb $24A_3$ showed the presence of cross-linked cytokeratin polypeptides in transplantable Morris hepatoma cells (Fig. 2, lane 2). These high molecular weight antigens were barely detectable in normal liver cells and cultured McA-RH7777 cells (Fig. 2, lanes 1 and 3).

Discussion

We have provided evidence that the method commonly used for cell fractionation of liver cells may generate artifacts due to cross-linking of proteins during homogenization and fractionation. The cross-linking reaction observed in our experiments was enhanced by Ca^{2+}, and inhibited by EGTA and iodoacetamide, suggesting the reaction was mediated by the action of Ca^{2+} and sulfhydryl dependent enzymes; possibly by tissue transglutaminases. This was further supported by the inhibition of cross-linking by primary amines (e.g. putrescine).

Transglutaminases (TG; EC2.3.2.13) are a family of enzymes which catalyze the formation of an isopeptide bond through an acyl transfer reaction between a peptide-associated glutamine and a primary amine or the ϵ-amino group of certain peptide-bound lysine residues (13). The hepatocyte is known to be rich in TG activity, but its function is still unknown (14). Tissue TG has been found to cause the covalent cross-linking of a number of membrane proteins into a very high molecular weight material which does not enter polyacrylamide gels (15). Hepatocellular cytokeratins were reported to be good substrates for TG (16). Glass et al. (17) reported that artifactual cross-linking of nuclear lamina proteins, which form intermediate type filaments in the nucleus and whose sequences are highly homologous to cytoplasmic intermediate filaments (18), would occur during chromatin preparation of Novikoff hepatoma cells in the presence of Ca^{2+} due to intrinsic nuclear transglutaminase.

Various enzymes are involved in many kinds of post-translational modification processes, which are organized and regulated within the living cells. It is very easy to speculate that enzymatic and non-enzymatic modifications of proteins, such as proteolysis and cross-linking, would occur immediately after the cell destruction. However, it seems that little attention has been paid by investigators to avoid artifactual cross-linking and aggregate formation during cell fractionation processes compared to the attention to proteolysis. Tyrrell et al. (19) reported that TG activity was not detectable in liver homogenates prepared in Ca^{2+}-free buffer. Contamination of Ca^{2+} from blood seems to be a major factor for these artifactual TG-induced cross-linking during homogenization, because liver and hepatoma tissue are especially blood-rich. Extensive perfusion of the liver with Ca^{2+}-free solution was shown to be effective in decreasing these artifacts (data not shown).

The results reported in this paper also confirmed our early observation of the presence of cross-linked cytokeratin polypeptides in transplanted rat

hepatoma cells (7). Several reports suggested that TG-induced cross-linking and aggregates of structural proteins could be a consequence of cell injury (20,21). Zatloukal et al. (16) speculated that TG-induced cross-linking of cytoskeletal components in hepatocytes may occur during liver cell injury associated with increased intracellular Ca^{2+} concentrations. Disorganization of the cytoskeleton filament network associated with the formation of aggregates of keratins is recognized in various liver diseases such as alcoholic hepatitis and hepatoma (4). Alcoholic hyaline or Mallory bodies, which have been used as histologic markers for these liver diseases, are now known as a aggregate of cytokeratins (4,22). Cross-linked cytokeratins were not detectable in the cultured hepatoma cells. The transplantation of hepatoma cells into the muscle tissue may cause metabolical alteration and the presence of cross-linked cytokeratins may be an indication of degenerative processes of tumor cells.

Alternatively it is possible that the cross-linking of cytokeratins may play an important role in specific cellular processes. It is well recognized that intermediate filaments are directly linked to nuclear matrix and desmosomes and also interact with various cytoplasmic organelles (3). Cytokeratins cross-linked to each other or with another cytoplasmic components may have some functional importance. Physiological meanings of cross-linked cytokeratins remain unanswered.

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