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Cadmium toxicity on cultured neonatal rat hepatocytes: Biochemical and ultrastructural analyses

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Summary. The effects of cadmium exposure on the protein secretory functions of cultured neonatal rat hepatocytes were analyzed by both two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and electron microscopy. [^{35}S]Methionine-labelled protein secretion was significantly depressed by cadmium exposure in a dose-dependent manner (1, 10 and 100μM). Protein secretory patterns resolved by 2D-PAGE and analyzed by autoradiography showed that besides albumin and transferrin, three polypeptide spots decreased their radiolabelling intensities, whereas four spots appeared due to cadmium exposure. Ultrastructural alterations in cultured neonatal rat hepatocytes induced by cadmium exposure were characterized by condensation of the nuclear chromatin, appearance of intra-nuclear inclusions, decrease in number of microvilli, increase in number of intra-mitochondrial granules and transformation of rough endoplasmic reticulum to cytoplasmic vesicles in a dose-dependent manner. Both biochemical and ultrastructural findings indicate that cadmium adversely affects the protein secretory functions of cultured neonatal rat hepatocytes.

Key words: Cadmium, Toxicology, Electrophoresis, Electron microscopy, Hepatocyte

Introduction

Environmental pollutions derived from cadmium are one of the serious problems in our daily life. It has been known that cadmium accumulates preferentially in the liver and causes dysfunctions of this organ (Flick et al., 1971; Friberg et al., 1974; Din and Frazier, 1983). A number of biochemical studies have investigated the hepatotoxicity of cadmium in *in vivo* experiment (Shaikh and Lucis, 1972; Webb, 1972; Whanger, 1973; Cook et al., 1974; Colucci et al., 1975; Hoffmann et al., 1975; Faeder et al., 1977). Also the establishment of techniques of hepatocyte isolation and culture has made possible various progressions in cadmium toxicity studies, since these cultured cells can maintain structural and functional integrity of the intact liver for several days (Stacey and Klaassen, 1981; Santone and Acosta, 1982; Müller and Ohnesorge, 1984; Sorensen and Acosta, 1984). However, these cultured hepatocytes are always derived from adult animals, and no attempts have been made on neonatal ones.

Recently, Mitane et al. (1987a,b) have showed that cadmium causes an inhibition of protein secretion in cultured adult rat hepatocytes. However, no biochemical studies have been concerned with the qualitative changes in protein secretions of cultured rat hepatocytes due to cadmium exposure. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is one of the most effective analyses to estimate the whole feature of protein secretion patterns of various organs (O'Farrell, 1975).

In the present study, effects of cadmium on the pattern of [^{35}S]methionine-labelled proteins secreted from cultured hepatocytes prepared from neonatal rats were analyzed by 2D-PAGE and autoradiography. In addition, ultrastructural observations of cadmium-exposed cultured neonatal rat hepatocytes were done to correlate with cadmium toxicity in protein secretory functions.

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Materials and methods

Preparation and culture of neonatal rat hepatocytes

Isolated rat hepatocytes were prepared from neonatal male Wistar rats (0 days old) by the collagenase shaking methods (Howard and Pesch, 1968) with some modifications. Livers were removed and placed in Ca^{2+}-free Hanks' Balanced Salt Solution (HBSS; Sigma,

St. Louis, U.S.A.). The tissue was minced with scissors and incubated for 15min at 37°C in a shaking water bath (70 oscillation/min). Minced tissues were collected on gauze by filtration and incubated with 0.1% collagenase solution (Hepatocyte Isolation type IV, Wako, Osaka, Japan) in HBSS for 30 min in the above mentioned shaking condition. Isolated cells were filtered through 100μm and 50μm mesh filters, whashed three times by slow centrifugation (1st, 1min at 100 x g; 2nd, 1min at 75 x g; 3rd, 1min at 50 x g), and resuspended in Eagle's Minimum Essential Medium (MEM; Sigma) containing 10% fetal bovine serum (Gibco, Chargin Falls, U.S.A.), non-essential amino acids (0.1mM), and antibiotics (penicillin 100u/ml) and streptomycin 100μg/ml). Hepatocytes were plated into collagen-coated plastic wells (17 x 16 mm; Corning, New York, U.S.A.) at a density of 4×10^5 cells/ml, allowed to attach to the dish for 2hr at 37°C in a humidified atmosphere of 5% CO_2 in air. The, free cells and medium were removed from the wells and attached hepatocytes were cultured in the fresh medium together with the above supplements for 24hr. The cell viability in the present study was about 90% by the trypan blue exclusion method.

[^{35}S] Methionine-labelling and cadmium exposure

Cultured neonatal rat hepatocytes (24 hours old) were washed twice with MEM containing 10% of the normal concentration of methionine (10% Met-MEM). [^{35}S]Methionine (American Radiolabeled Chemicals, St. Louis, U.S.A.; Specific acivity 1156Ci/mM) was added to a final concentration of 100μCi/ml. Cells were exposed for 4hr with or without cadmium sulphate (Wako) at concentrations of 1, 10 and 100μM in 10% Met-MEM. Three replicates were made for control and for each concentration of cadmium. The radioactive medium was aspirated, centrifuged at 15,600 x g for 5 min to remove cell breakdowns and debris, frozen at -70° C and lyophilized. Samples were reconstituted in 100μM lysis buffer (O'Farrell, 1975). Thrichloroacetic acid (TCA)-precipitable radioactivity was determined as reported previously (Ueda et al., 1988). Samples containing 1,000,000cpm radioactive materials were analyzed by 2D-PAGE as described previously (Ueda et al., 1990). The method for determining comparative polypeptide radioactivity has been previously reported (Ueda et al., 1988).

After removing radioactive medium, cells were washed twice with HBSS and lysed in 100μl lysis buffer. The cell lysates were assayed for protein concentration by the method of Peterson (1977).

Electron microscopy

Cultured neonatal rat hepatocytes (24 hours old) were washed twice with MEM, and exposed for 4hr with or without cadmium sulphate as described above. At the end of the experiment, cells were fixed with 2% paraformaldehyde-1% glutaraldehyde in 0.1M phosphate buffer (PB, pH7.2) for 2hr, rinsed with 0.1M PB containing 10% sucrose for 2hr, and postfixed with 1% osmium tetroxide in 0.1M PB for 1hr at 4° C. Finally, they were dehydrated by the graded ethanol series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife on a Porter-Blum MT-1 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a JEM 100CX electron microscope.

Statistics

Statistical comparisons were made by Student's t-test with $P < 0.01$ as the limit of significance.

Results

Effects of cadmium on protein secretion of cultured neonatal rat hepatocytes

Cultured neonatal rat hepatocytes were incubated with [^{35}S] methionine in 10% Met-MEM for 4hr in the continuous presence or absence of various concentrations of cadmium (1,10 and 100μM). [^{35}S]Methionine-labelled proteins released into the incubation medium, which were expressed as TCA-precipitable radioactivity per mg cellular protein, were significantly depressed by the cadmium exposure in a dose-dependent manner (Fig. 1; $p < 0.01$, 10μM and 100μM vs. control).

The same amounts of [^{35}S]methionine-labelled secretory proteins (1,000,000cpm) for the 4 samples were resolved by 2D-PAGE and analyzed by autoradiography. Besides albumim (Alb) and transferrin (Tf), whose positions were detected by western blotting technique using anti-rat Alb and anti-rat Tf sera (data not shown), several polypeptide spots were observed in both control (Fig. 2a) and in the experiments with various concentraions of cadmium (Fig. 2b, 1μM; Fig. 2c, 10μM; Fig. 2d, 100μM). Among them, radioactivity levels of L70 (Mr70,000/pI 4.65). L68 (68,000/4.66) and

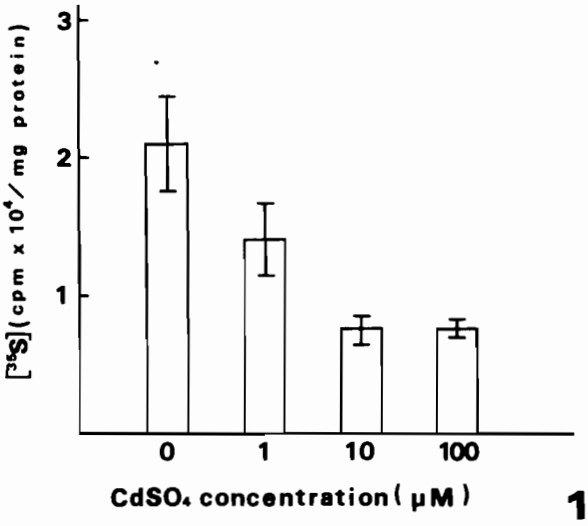


Fig. 1. Effect of various concentrations (1, 10, 100μM) of cadmium on total protein secretion in cultured neonatal rat hepatocytes. The cells (24 hours old) were incubated with [^{35}S]methionine for 4hr with or without cadmium sulphate. Radiolabelled secretory protiens and cellular proteins were determined as described in Materials in Materials and methods. Each bar represents the mean and SD of three replicates.

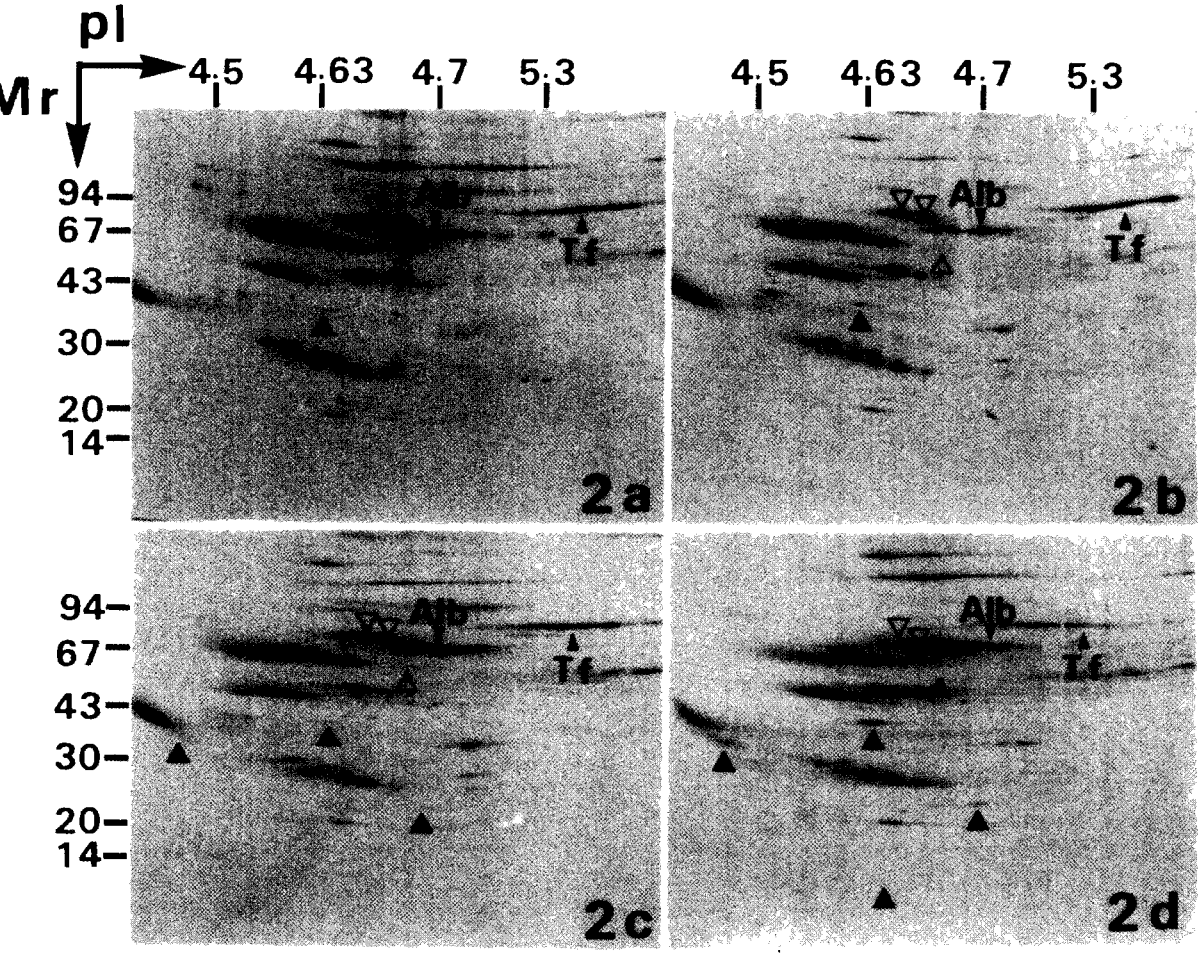


Fig. 2. Autoradiograms showing radiolabelled proteins (1,000,000cpm) accumulated in the medium of cultured neonatal rat hepatocytes in the absence (a) or presence of various concentrarions of cadmium sulphate (1μM, b; 10μM, c; 100μM, d). Besides albumin (Alb) and transferrin (Tf), three polypeptide spots decrease their radiolabelling intensity (open arrowhead), whereas four spots increase their levels of radioactivity (closed arrowhead).

L60 (60,000/4.68) were decreased in 1μM (Fig. 2b) and 10μM (Fig. 2c), and hardly detected in 100μM cadmium-exposure (Fig. 2d). On the contrary, L43 (43,000/4.63), L38 (38,000/4.45) and L23 (23,000/4.78), which were not observed in control (Fig. 2a), became visible in 10μM (Fig. 2c) and 100μM cadmium-exposure (Fig. 2d). Moreover, L12 (12,000/4.64) was only detected in 100μM cadmium-exposure (Fig. 2d).

Table 1 provides comparative cpm values of albumin and transferrin that confirm subjective estimates of [^{35}S] methionine-labelling intensity in the presence or absence of cadmium. These findings were in accordance with the result of total protein secretion demonstrated in Fig. 1.

Effects of cadmium on ultrastructure of cultured neonatal rat hepatocytes

Cultured cells were prepared at a purity of more than 90% of hepatocytes by electron microscopic analysis. The contaminating cells were mostly fibroblasts which were readily distinguished from hepatocytes by their elongated shape and the absence of microvilli. Cultured neonatal rat hepatocytes that were placed for 24hr in medium containing 10% fetal bovine serum and 4hr in serum-deprived medium were used as control (Fig. 3a). These cells were characterized by many microvilli, nuclei with a small ,amount of condensed chromatin, moderate electron-dense mitochondria, and dispersed rough endoplasmic reticulum.

Cultured neonatal rat hepatocytes that were exposed to cadmium for 4hr showed various ultrastructural alterations in a dose-dependent manner. In 1μM cadmium-exposed cells, microvilli were markedly decreased in number and some of them were trapped into a cell body in the form of a loop (Fig. 3b). Nuclei became irregular in shape and peripheral chromatin was obviously condensed. Intra-mitochondrial granules seemed to increase in number (Fig. 3b). In 10μM cadmium-exposed cells, microvilli were sparse but drawing loop structures were still observed (Fig. 3c). Nuclei became indented in shape and a large volume of condensed chromatin occupied the nuclear bodies. Mitochondria increased their electron density and intra-mitochondrial granules were clearly increased in number. Rough endoplasmic cisterns were observed to loosen the ribosome and transform into small vesicles (Fig. 3c). In 100μM cadmium-exposed cells, no microvilli were observed and several large cytoplasmic vacuoles were present in the cytoplasm (Fig. 3d). Most of the

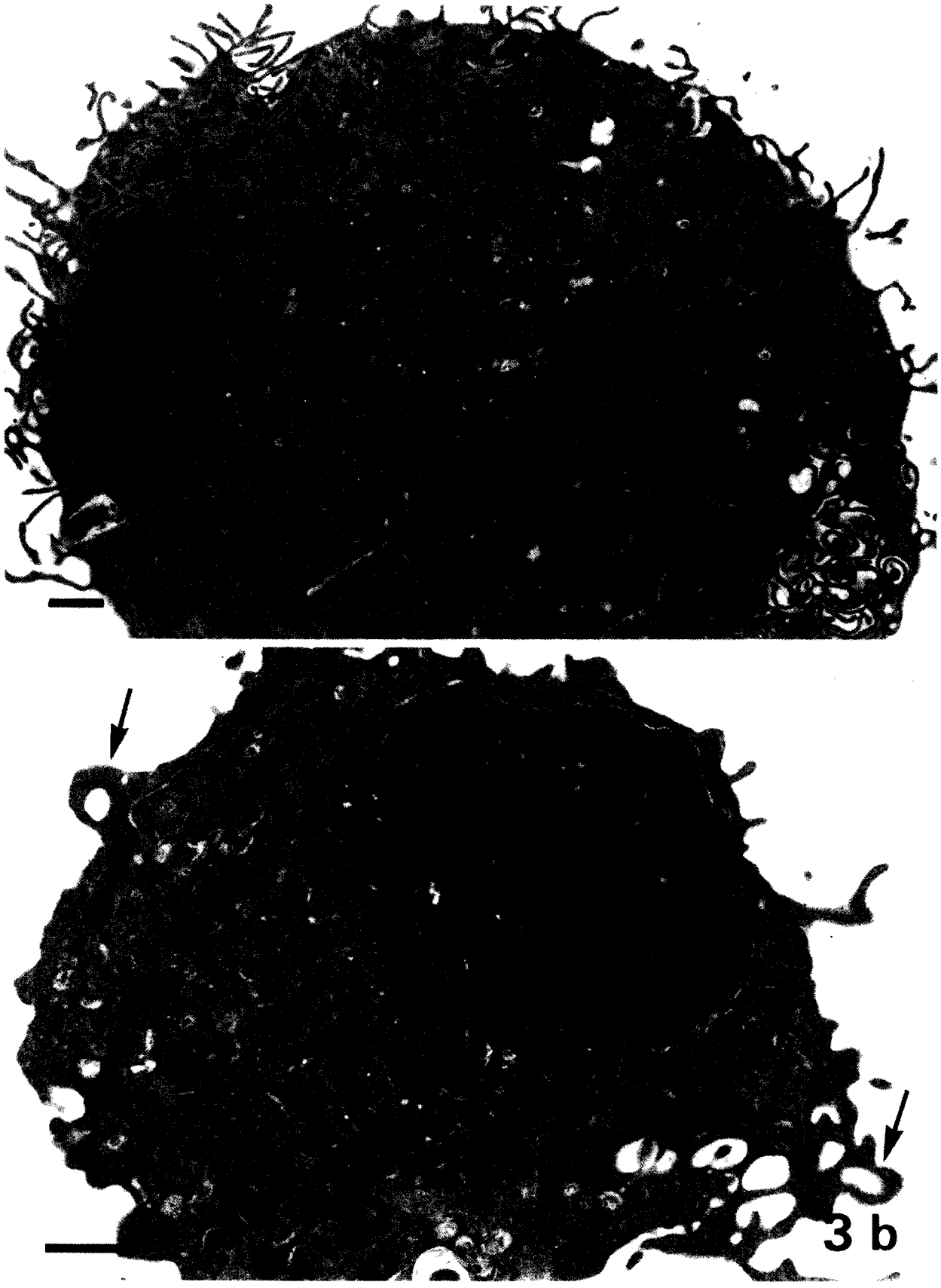
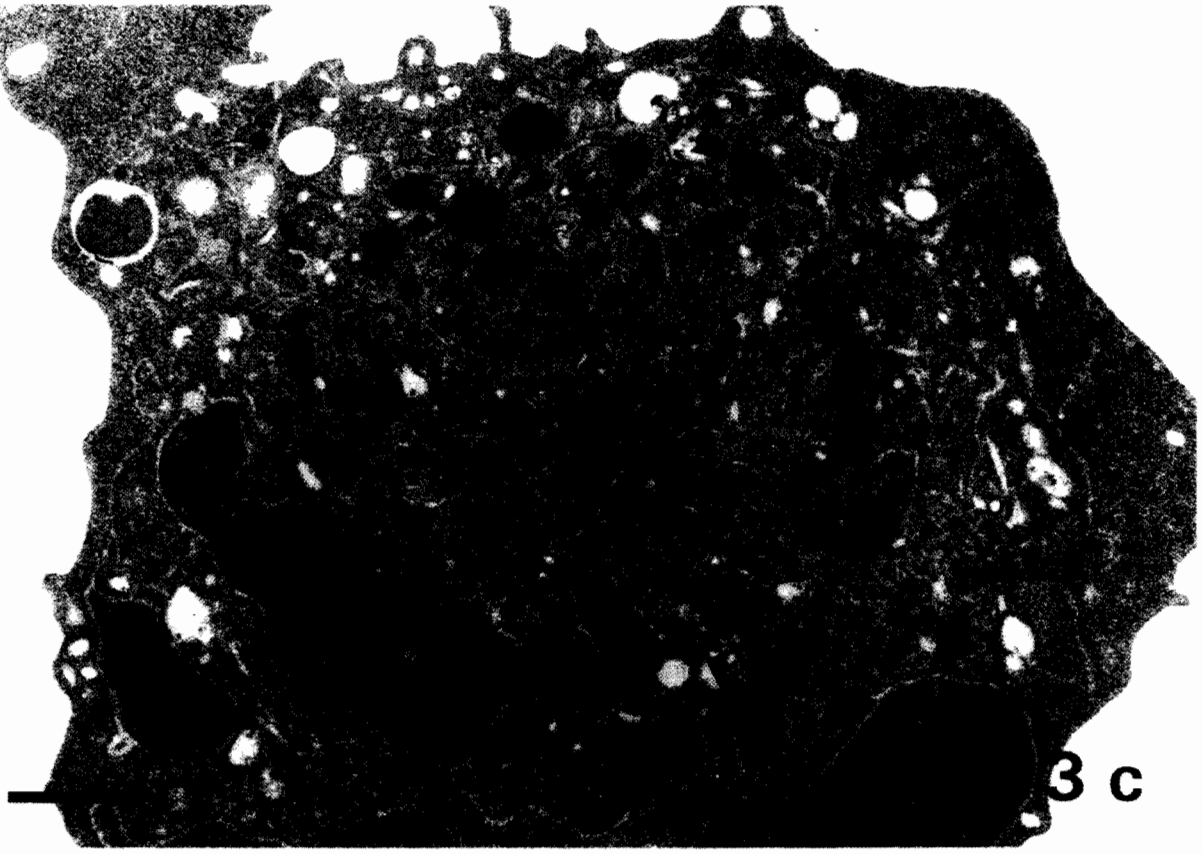


Fig. 3. Electron micrographs of neonatal rat hepatocytes cultured for 24hr with calf serum and for 4hr in serum-deprived medium without any addition (Fig. 3a) and with cadmium sulphate at a concentration of 1μM (Fig. 3b), 10μM (Fig. 3c) and 100μM (Fig. 3d). Arrows and arrowheads in Fig. 3b indicate trapped microvilli in the main cell body and intra-mitochondrial granules, respectively. Arrows and arrowheads in Fig. 3c illustrate degradation of rough endoplasmic reticulum to cytoplasmic vesicles and intra-mitochondrial granules, respectively. CV and IN in Fig. 3d demonstrate cytoplasmic vacuoles and intra-nuclear inclusions, respectively. Bars, 1μm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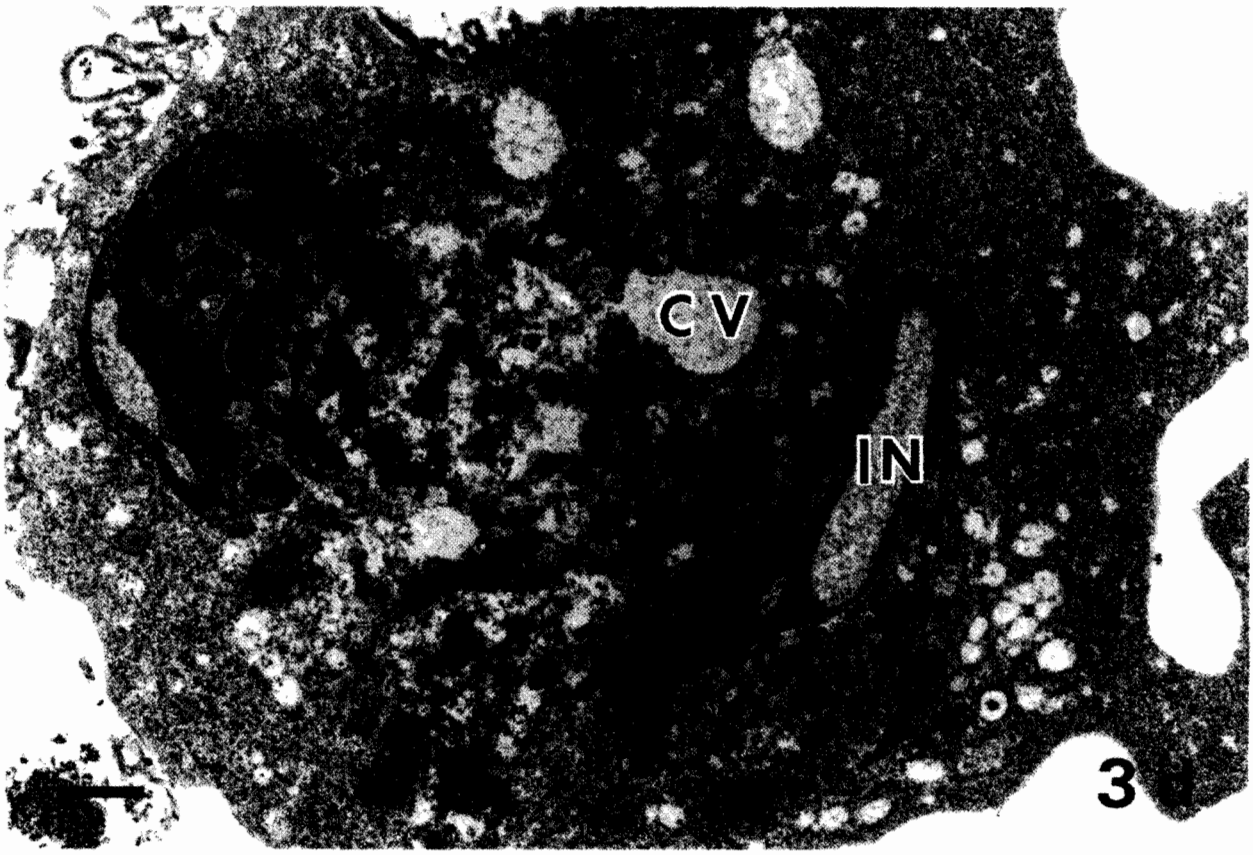
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Table 1. Comparative radioactivity of [^{35}] methionine-labelled albumin and transferrin.

Cadmium concentration (μM)	Albumin (cpm)	Transferrin (cpm)
0	592	700
1	333	530
10	138	266
100	126	86

Values for equivalent surface area of radiolabelled spots (albumin and transferrin) were determined by pairs of electrophoretic protein maps.

mitochondria were swollen in shape and few intramitochondrial granules were detected. Rough endoplasmic reticulum was hardly observed. Some of the indented nuclei contained large intra-nuclear inclusions (Fig. 3d).

Discussion

It has been shown that cadmium inhibits total protein secretion (Mitane et al., 1987a) and secretions of albumin and transferrin (Mitane et al., 1987b) from cultured adult rat hepatocytes. In the present study, we found the depression of total protein secretion as well as secretions of albumin and transferrin by cadmium exposure in cultured hepatocytes prepared from neonatal rats. To date, although many biochemical studies have involved the quantitative changes in protein synthesis and secretion of cadmium-treated rat liver in both *in vivo* and *in vitro* experiments (Din and Frazier, 1983), no biochemical studies have been carried out to demonstrate the qualitative changes in protein secretion of cadmium-exposed rat liver.

The same amounts of radiolabelled proteins for the 4 samples were analyzed by 2D-PAGE to compare the direct difference between the control and various concentrations of the cadmium exposure. The present study demonstrated for the first time that protein secretory patterns were changed by cadmium exposure in cultured neonatal rat hepatocytes. The present electrophoretic analysis is considered to be reliable since basic protein secretory patterns were not altered even in 100μM cadmium-exposed cultured neonatal rat hepatocytes. By cadmium exposure, three polypeptide spots as well as albumin and transferrin decreased their levels of radioactivity. On the contrary, four spots became visible in higher concentrations of cadmium. Since these spots have never been observed in cellular proteins analyzed by 2D-PAGE (Hirano, unpublished data), their synthetic and secretory pathways might be altered by cadmium exposure. The functional significance of both appearance and disappearance of cadmium-dependent polypeptides remains to be elucidated.

There was a good correspondence between ultrastructural alterations and changes in protein secretory functions in cultured neonatal rat hepatocytes. In the hepatocytes, the biochemical nature of secretory proteins is determined in the nucleus where chromatin plays important roles in constructions of protein products. The ribosome attached to the endoplasmic reticulum is the site where secretory protein synthesis is initiated. Then, secretory proteins are transported through the Golgi apparatus to the plasma membrane and finally secrete by exocytosis (Farquhar, 1983; Fawcett, 1986). A number of ultrastructural studies have demonstrated cadmium-induced hepatocyte alterations; nuclear heterochromatin condensation, loss of microvilli, degenerative mitochondrial abnormalities, deterioration of rough endoplasmic reticulum and cytoplasmic vacuolation (Faeder et al., 1977; Puvion and Lange, 1980; Morselt et al., 1983; Sorensen and Acosta, 1984).

Nuclear changes in the present observations were noticed even in a low concentration of cadmium exposure, and these changes might be related to a disturbance in either messenger RNA production or ribosomal RNA synthesis. The transformation of rough endoplasmic reticulum to cytoplasmic vesicles also suggested that ribosomal RNA synthesis was disturbed by cadmium exposure. The dose-dependent alteration of microvilli was closely related to the depression of total protein synthesis. But further detailed cytological investigations should be carried out to interpret which organella are responsible for the qualitative changes in protein secretion.

In summary, the present study indicated that protein secretory functions of cultured neonatal rat hepatocytes were adversely affected by cadmium exposure. It is worthwhile speculating that the changes in protein secretory mechanisms altered by cadmium treatment is involved in the clarification of cadmium toxicity in rat liver. The functional significance of altered secretory polypeptide spots is under investigation by several molecular approaches.

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