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Cleavage of nucleolin and AgNOR proteins during apoptosis induced by anticancer drugs in human salivary gland cells

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BACKGROUND: To investigate the behavior of nuclear proteins in apoptosis induced by anticancer drugs in cultured human salivary gland (HSG) cells.

METHODS: Dynamic alternations of nucleolin and argyrophilic nucleolar organizer region (AgNOR) proteins in anticancer drug-induced apoptosis of HSG cells and in a cell-free apoptotic system were examined using Western blot analysis and immunocytochemical method.

RESULTS: The 110-kDa form of nucleolin and AgNOR protein decreased and the 80- and 95-kDa forms appeared during apoptosis in HSG cells and in a cell-free apoptotic system. In addition, the induction of DNA ladder formation coincided with the appearance of alternation of nucleolin and AgNOR proteins in a cell-free apoptosis. Nucleolin diffusely spread out into the nuclear material in the apoptotic body of HSG cells.

CONCLUSIONS: The present results indicate that alternations of nucleolin and AgNOR proteins are associated with the induction of DNA fragmentation and the final active phase of apoptosis induced by anticancer drugs in malignant salivary gland cells.

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Keywords: argyrophilic nucleolar organizer region; anticancer drugs; apoptosis; human salivary gland cells; nucleolin

Introduction

Apoptosis is a morphologically and biochemically distinct mode of cell death that occurs during embryogenesis, carcinogenesis, cancer treatment, or immune and toxic cell killing as well as cell proliferation (1, 2). Distinctive morphologic features of apoptosis are cell rounding, cell shrinking, and condensation of the nucleus (1, 2). The alternation of nuclear proteins also appears to be related to apoptosis. Degradation of nuclear matrix-associated proteins such as histone H1 (3), ribonucleoprotein A1 (4), lamin B (5), and NuMA (6) may have an important role in apoptosis execution. Morphologic changes during apoptosis are reportedly induced by the alteration of various nuclear proteins, including lamin B (5), NuMA (6), argyrophilic nucleolar organizer region (AgNOR) proteins (7), and nucleolin (8).

Our previous reports have demonstrated that nucleolin and the 110-kDa form of AgNOR proteins decreased while an 80-kDa protein, cleaved from the 110-kDa form, appeared during apoptosis induced by okadaic acid or calyculin A in cultured osteoblastic cells (7, 8). Okadaic acid acts in cytoplasm and/or on the cell surface through the Fas/Fas ligand system to induce apoptosis (9, 10). However, it has been suggested that anticancer drugs directly attack the double- and single-strands of DNA (11) to induce cytotoxicity and apoptosis in the cells. The characterizations of nucleolin and AgNOR proteins in apoptotic cells induced by these agents are not yet known.

In the present study, the alternation of nucleolin and AgNOR proteins during apoptosis induced by anticancer drugs in cultured human salivary gland (HSG) cells was investigated.

Materials and methods

Materials

α-Modification of Eagle minimum essential medium (α-MEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). Plastic tissue culture dishes were purchased from Iwaki (Chiba, Japan). Cisplatin and mitomycin C were purchased from Wako Chemical Co. (Osaka, Japan). Stock solutions (10 mM), prepared in phosphate-buffered saline (PBS), were diluted to the appropriate concentrations with medium and added to the culture media to obtain the appropriate final concentrations. Ribonuclease (RNase) and Hoechst 33342 dye were purchased from Sigma (St Louis, MO, USA). Proteinase K was obtained

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from Merck (Darmstadt, Germany). Other materials used were of the highest grade commercially available. HSG cells that neoplastic epithelial duct cell line established from an irradiated HSG, were provided by Dr Mitsunobu Sato (12). The cells were grown in plastic dishes containing α-MEM supplemented with 10% FBS at 37°C in humidified atmosphere of 5% CO_2 and 95% air. The cells were subcultured every 5 days using 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS free of Ca^{2+} and Mg^{2+}.

Determining cell viability with the WST-1 assay
Untreated or anticancer drug-treated HSG cells were cultured at 2×10^4 cells/ml in a 96-well plate. A sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay was used because this compound produces a highly water-soluble, highly visible formazan dye. Cell viability was assessed with the WST-1 assay (13) after anticancer drug treatment. Samples were cultured and assayed in triplicate, and the error bars represent the standard error of the data.

DNA isolation and agarose gel electrophoresis
The DNA fragmentation was detected using a previously reported method (7–10). In brief, purification of DNA from cultured cells was accomplished after two washes in PBS followed by lysis in cold 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 0.5% Triton X-100. After lysis, the debris was removed by centrifugation at 15 000 *g* for 20 min. A solution of DNAse-free RNAse (Sigma) was added to the lysates at a final concentration of 40 μg/ml; the resulting mixture was incubated with gentle shaking for 1 h at 37°C. Proteinase K (Merck) was added to the RNAse-treated lysates at a final concentration of 40 μg/ml. The lysates were further incubated for 1 h at 37°C with gentle shaking. The DNA in the supernatant was extracted, precipitated with ethanol, and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA was quantified by UV spectrophotometry. Electrophoresis was performed in a 2.0% agarose gel containing 0.5 μg/ml ethidium bromide.

Apoptosis assays, immunocytochemistry, and AgNOR staining
The cells were plated on sterile 18-mm round glass coverslips placed in 35-mm plastic dishes and cultured under various conditions. For immunocytochemistry, the cells on coverslips were washed three times with PBS, fixed with 3.7% formaldehyde for 10 min at ambient temperature, and permeabilized with methanol for an additional 20 min at −20°C. Subsequent procedures were conducted at ambient temperature. Non-specific binding sites were blocked with 4% bovine serum albumin (BSA) and 5% normal goat serum in PBS for 10 min in a humidified atmosphere. After a rinsing with cold PBS, the coverslips were incubated with 5 μg/ml of the immunoglobulin G (IgG) fraction of antinucleolin antibody connected with fluorescein isothiocyanate (FITC)-conjugated antimouse IgG (diluted 1:100; Santa Cruz, CA, USA) in 4% BSA for 45 min. Three washes with PBS-Tween over a 15-min period. Finally, the cells were incubated with Hoechst 33342 (10 μg/ml) for 10 min, washed with PBS-Tween as described above, and mounted with Gel/Mount™ aqueous mounting medium (Biomeda, Foster City, CA, USA). The AgNOR staining was performed according to the described procedure (7). After AgNOR staining, the cells were incubated with Hoechst 33342 (10 μg/ml) for 10 min at ambient temperature. After a rinsing with distilled water, the coverslips were mounted in aqueous mounting solution. The samples were examined under an Olympus microscope (model BX50: Olympus Corporation, Tokyo, Japan) equipped for epifluorescent illumination (Bx-FLA) and for photomicroscopy (PM-30). Photomicrographs were taken on Fuji Sensia™ 400 film (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Detection of nucleolin and AgNOR proteins
After appropriate periods of cultivation with or without anticancer drug treatment, the cells were washed twice with PBS and scraped into lysate buffer containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupepin, 2 μg/ml aprotinin, and 5 mM ethyleneglycoltetraacetic acid (EGTA) in PBS. The cells were then sonicated for 10 s, denatured in sample buffer, and heated in boiling water for 5 min. The protein concentration was evaluated by using a commercial reagent (Protein Assay Reagent™; Bio-Rad, Hercules, CA, USA); for this assay, the cell lysate was diluted to 1 mg/ml with lysate buffer before the addition of 5X sample buffer. The following procedures were performed at ambient temperature. About 10 μg of each sample and pre-stained molecular weight markers (Gibco-BRL, Gaithersburg, MD, USA) were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to polyvinylidene difluoride transfer membranes that were incubated for 2 h in a blocking solution containing 5% skim milk and 0.1% Tween 20 in PBS (PBS-Tween). The membranes were washed briefly in PBS-Tween and incubated for 1 h with antinucleolin antibody diluted 1:100. The membranes were then washed four times within 30 min in PBS-Tween on a rotary shaker. The washed membranes were incubated with horseradish peroxidase (HRP)-conjugated antimouse IgG (diluted 1:2500) for 1 h. The membranes were washed as described above and proteins recognized by the antibody were visualized with an ECL detection kit (Pharmacia Biotec, Uppsala, Sweden) used according to the manufacturer's directions. For AgNOR staining, the membranes were treated twice with ethanol for 10 min. The membranes were incubated for 20 min in a mixture of solution A (2 g gelatin in 100 ml water containing 1% formic acid) and solution B (0.5 g/ml $AgNO_3$ in distilled water) with gentle shaking in the dark. Staining was stopped by thoroughly rinsing the membranes with distilled water.

Cell fractionation and detection of nucleolin and AgNOR proteins
Cells cultured in 90 mm plastic dishes were washed twice with PBS, scraped into PBS, pelleted at 3000 *g*, and

resuspended in hypotonic buffer (20 mM HEPES, pH 7.2, 10 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 0.5 mM EDTA). Cells were allowed to swell for 10 min in ice before lysis by addition of 0.1% NP-40 and 100 mM potassium acetate. After 5 min on ice and vortexing, nuclei were pelleted by centrifugation for 10 min at 8000 *g*, resuspended in lysate buffer containing 1 mM DTT, 1 mM PMSF, 1 mg/ml leupeptin, 2 mg/ml aprotinin, and 5 mM EGTA in PBS, and termed the nuclear fraction, whereas the supernatant was centrifuged for 60 min at 100 000 *g* and the supernatant was termed the cytosolic fraction. Each sample was used for Western blot analysis with antinuleolin antibody and for the detection of AgNOR proteins as described.

Identification of nucleolin and AgNOR proteins in a cell-free apoptosis system

A cell-free apoptosis system was prepared using a previously reported method (7, 8). In brief, the cytosolic fraction was prepared to a final protein concentration of 6 mg/ml with lysate buffer. The 3×10^6 nuclei prepared from non-apoptotic HSG cells were incubated for 2 h at 37°C with 30 μl of cytosolic fraction from anticancer drug-treated HSG cells or untreated cells. Agarose gel electrophoresis analysis of DNA was performed. To detect nucleolin and AgNOR proteins in a cell-free apoptosis system, 5X SDS sample buffer was added to the nuclei–cytosol mixtures and vortexed lightly. The reaction mixtures were boiled for 5 min and centrifuged at 10 000 *g* for 10 min; the supernatant was assayed by SDS-PAGE and transblotted. The transferred proteins were stained with the antinucleolin antibody and AgNOR protein staining technique mentioned above.

Results

Anticancer drugs-induced apoptotic cell death in HSG cells

Two kinds of anticancer drugs significantly increased the cell death in HSG cells in a dose-dependent manner as seen with phase-contrast microscopy and with the WST-1 assay (data not shown). The anticancer drugs-induced cytotoxicity in a dose-dependent manner up to 100 μM (data not shown).

The DNA ladder formation analysis showed that a DNA fragmentation pattern in multiples of 185–200 bp was formed from cells treated with cisplatin- and mitomycin C-treated HSG cells (Fig. 1a,b). In anticancer drugs-treated HSG cells, DNA ladder formation was shown to be both dose-dependent (Fig. 1a,b) and time-dependent (data not shown).

Detection of nucleolin and AgNOR proteins and their cleaved form in HSG cells

Figure 2 shows the results of Western blot analysis of nucleolin (Fig. 2a,c) and AgNOR proteins (Fig. 2b,d) in cell lysates obtained from the control and anticancer drug-treated HSG cells. Using Western blot analysis with antinucleolin antibody, the control HSG cells were immunopositive and had an estimated molecular weight of 110 kDa. The staining intensity of the 110-kDa protein in the lysate prepared from the cisplatin-treated cells decreased in a dose-dependent manner. Proteins in 80- and 95-kDa forms were also detected in the lysate of cells treated with cisplatin, and their staining intensities increased in a dose-dependent manner up to 100 μM, unlike that of the control cells (Fig. 2a). Using AgNOR staining with modified Western blot analysis, although several minor bands are visible, four major AgNOR-positive bands with estimated molecular weights of 110, 37, 32, and 30 kDa were observed in the lysate obtained from the control cells (Fig. 2b). The reaction intensity of the 110-kDa protein was decreased in the lysate prepared from cisplatin-treated HSG cells while 80- and 95-kDa proteins appeared and gradually increased in the same preparations (Fig. 2b). To confirm that the cleavage of nucleolin and AgNOR proteins does not occur only in cisplatin-treated HSG cells, we stained proteins from HSG cells treated with various concentrations of mitomycin C with the antinucleolin antibody and AgNOR staining. The intensity of a 110-kDa band also decreased

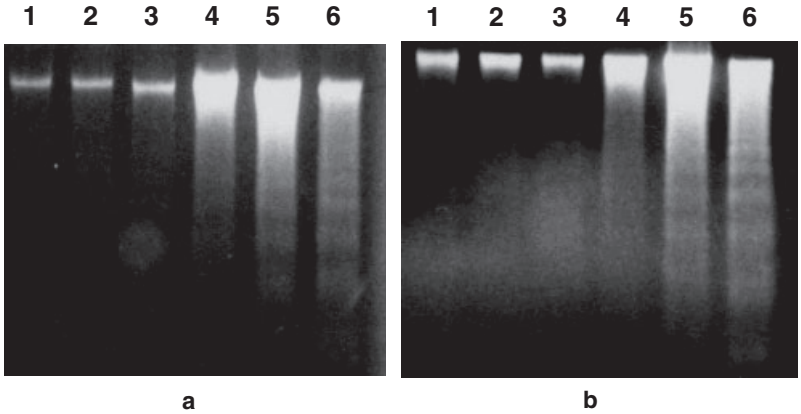

Figure 1 Effects of cisplatin or mitomycin C in human salivary gland (HSG) cells. DNA ladder formation in HSG cells treated with cisplatin (a) or mitomycin C (b). Subconfluent HSG cells were exposed for 24 h with various concentrations both of two. Lane 1, untreated control cells; lane 2, cells treated with 0.5 μM cisplatin or mitomycin C; lane 3, 5 μM; lane 4, 25 μM; lane 5, 50 μM, and lane 6, 100 μM.

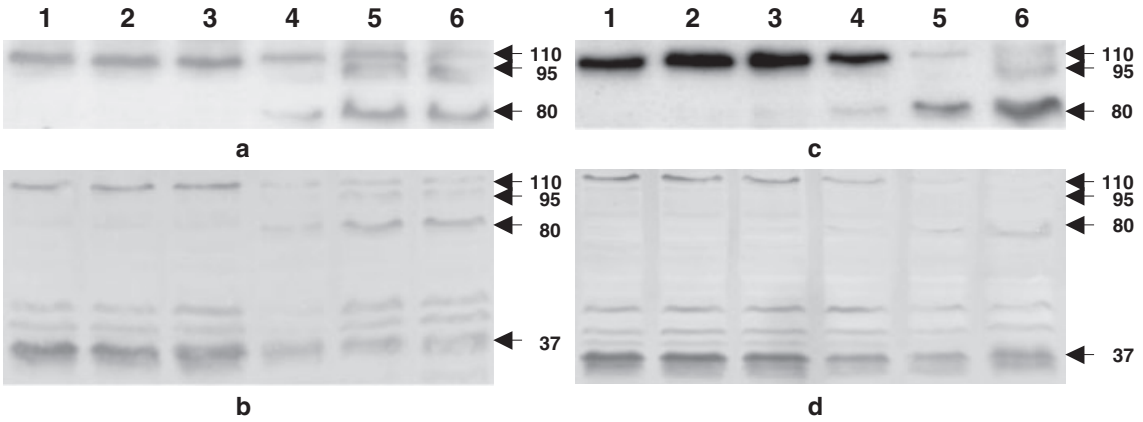

Figure 2 Identification of nucleolin (a and c) and AgNOR proteins (b and d) in human salivary gland (HSG) cells using Western blot analysis. Subconfluent cells were treated for 24 h with various concentrations of cisplatin (a and b) or mitomycin C (c and d) and the proteins were subsequently prepared. The staining method is described in the Materials and Methods section. The molecular weight is indicated along the right side (kDa). Lane 1, untreated control cells; lane 2, cells treated with 0.5 μM cisplatin or mitomycin C; lane 3, 5 μM; lane 4, 25 μM; lane 5, 50 μM, and lane 6, 100 μM.

and those of the 80- and 95-kDa bands appeared in the lysates of HSG cells that had been treated with mitomycin C (Fig. 2c). The 110-kDa band decreased and the 80- and 95-kDa bands increased in a dose-dependent manner (Fig. 2d).

Nucleolin and AgNOR proteins during apoptosis in a cell-free system

Figure 3a shows that the apoptotic cytosolic fraction-induced DNA ladder formation in nuclei prepared from the normal HSG cells in time course. The DNA ladder formation in cell-free apoptosis was increasing in time-dependent fashion. However, the cytosolic fraction prepared from the control cells did not induce DNA fragmentation in nuclei obtained from HSG cells. Regarding the alternation of nucleolin in a cell-free apoptotic system in time course, nucleolin was detected in the proteins obtained from intact nuclei of HSG cells incubated with the cytosol fraction of the control cells, just as with the *in vivo* apoptosis (Fig. 3b). The 80- and 95-kDa forms of nucleolin were detected and increasing at the same time coincided with appearance and increasing of DNA ladder formation in the cell-free system, and the staining intensity of the 110-kDa band decreased in the same preparation (Fig. 3b). Figure 3c also shows the alternation of AgNOR protein just as occurred with alternation of nucleolin *in vitro*. In addition, the cytosolic fraction prepared from the control cells mixed with cells exposed to the anticancer drugs did not induce DNA fragmentation and the cleavages of nucleolin in the nuclei obtained from HSG cells (data not shown).

Redistribution of nucleolin and AgNOR proteins in anticancer drug-induced apoptosis in HSG cells

The control cultures of HSG cells (Fig. 4a,b) did not show any apoptotic features. In the control cells, which have normal chromatin, when the nucleoli were intensively labeled with antinucleolin antibody, they appeared as dots in the nuclei, whereas the other cellular compart-

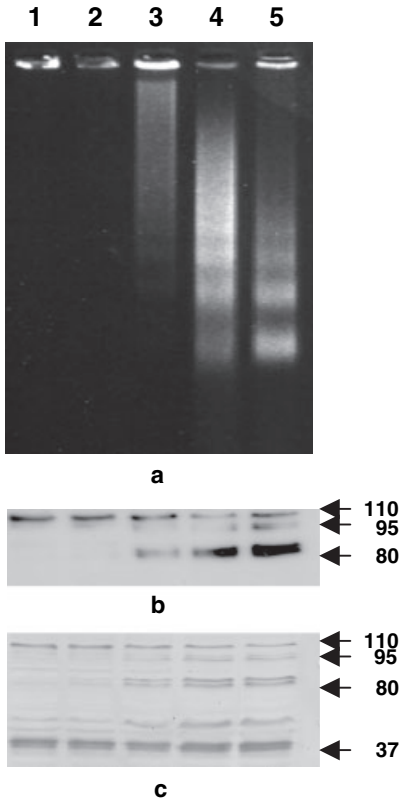

Figure 3 The DNA fragmentation (a) and alternation of nucleolin (b) and AgNOR proteins (c) in nuclei from human salivary gland (HSG) cells treated with cytosolic extracts from apoptotic HSG cells in time course. Intact nuclei were incubated with cytosolic fraction of control HSG cells or of treated for 14 h with 50 μM cisplatin. The DNA was extracted and electrophoresed through an agarose gel, yielding a ladder pattern (a). Each 20-μl reaction mixture described in the Materials and Methods section underwent Western blot analysis for nucleolin (b) or modified Western blot analysis for AgNOR proteins (c). The molecular weight is indicated along the right side (kDa). Lane 1, untreated control cells; lane 2, cells treated with 0 min; lane 3, 15 min; lane 4, 30 min; lane 5, 60 min, and lane 6, 120 min.

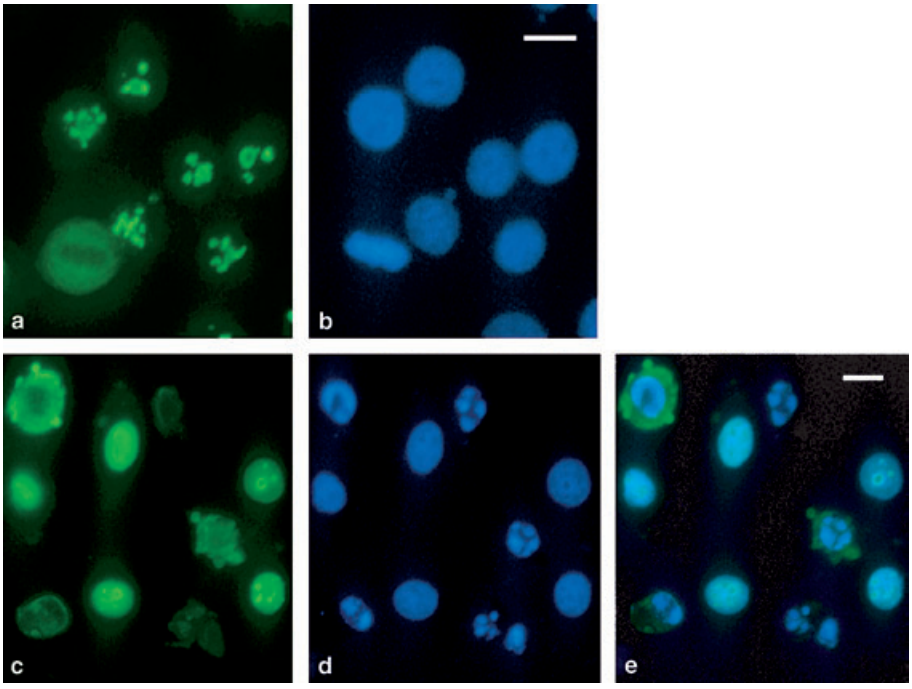

Figure 4 Identification of nucleolin in human salivary gland (HSG) cells. Apoptosis was induced by treatment with 50 μM cisplatin for 24 h. The staining methods are described in the Materials and Methods section. Untreated control cells were stained with antinucleolin antibody (a) or Hoechst 33342 (b). Cisplatin-treated cells were stained with antinucleolin antibody (c) or Hoechst 33342 (d), (e) is a superimposition of (c) and (d). The bar represents 10 μm.

ments were devoid of staining (Fig. 4a). In cisplatin-treated HSG cells, diffuse nucleolin was detectable within the nuclei of the apoptotic cell bodies (Fig. 4c,d). Superimposition confirmed that nucleolin was detected in the apoptotic body nuclei of HSG cells (Fig. 4e).

For the detection of AgNORs in HSG cells, the control and cisplatin-treated HSG cells were also fixed, permeabilized, and stained with silver nitrate and Hoechst 33342. In the control cells, the nuclei were intensively labeled with silver nitrate and were visible as dots in the nucleoli (Fig. 5a,b). The AgNORs were undetectable in the apoptotic HSG cell bodies, but diffuse AgNOR-positive staining was seen in whole apoptotic bodies (Fig. 5c,d). Furthermore, superimposed views confirmed that AgNORs had disappeared and diffuse AgNOR-positive staining appeared in the apoptotic nuclei of HSG cells (Fig. 5e).

In addition, we determined the subcellular localization of nucleolin in normal and apoptotic HSG cells using the Western blot technique. Cellular fractionation was done with HSG cells and proteins were prepared from each fraction. The 110- or 80-kDa band was detected in the nuclear fraction prepared from normal or apoptotic cells respectively (Fig. 6). The staining intensity of 110 and 80 kDa bands was undetectable level in the cytosolic fraction prepared from normal and apoptotic HSG cells (Fig. 6).

Discussion

In the present study, we investigated the alternations of nucleolin and AgNOR proteins during apoptosis induced by anticancer drugs in HSG cells using Western blot analysis and cytochemical method. The 110-kDa form of nucleolin decreased while the 80- and 95-kDa forms appeared during apoptosis induced by the two anticancer drugs in cultured HSG cells and in a cell-free apoptotic system. The induction of DNA ladder formation also coincided with the appearance of alternation of nucleolin and AgNOR proteins in a cell-free apoptosis. In addition, as shown by immunocytochemical analysis, nucleolin diffused into the nuclear material of the apoptotic body of HSG cells. The 110- and 80-kDa AgNOR proteins observed in our previous study (7) were speculated to be nucleolin on the basis of both molecular weight (14, 15) and the similarities of dynamic alternation in apoptotic cells.

Nucleolin is a phosphoprotein operating as a shuttle between the cytoplasm and nucleus (16) and may be involved in multiple functions such as cell proliferation, mitosis, and apoptosis (7, 8, 14–23). In the previous study (8) and the present study, we demonstrated that nucleolin disappeared or was redistributed in various kinds of apoptotic cells (18). When DNA ladder formation was detectable in the apoptotic cells, alternation of nucleolin was observed both *in vivo* and *in vitro*. Conversely, alternation of nucleolin was not observed with undetectable DNA ladder formation *in vivo* and *in vitro*. In addition, the induction of DNA ladder formation coincided with the appearance of alternation of nucleolin and AgNOR proteins in a cell-free apoptosis. Nucleolin appears to play an important role in mitosis (17, 18). The apoptotic morphologic features are similar to the hypercondensation of chromatin that

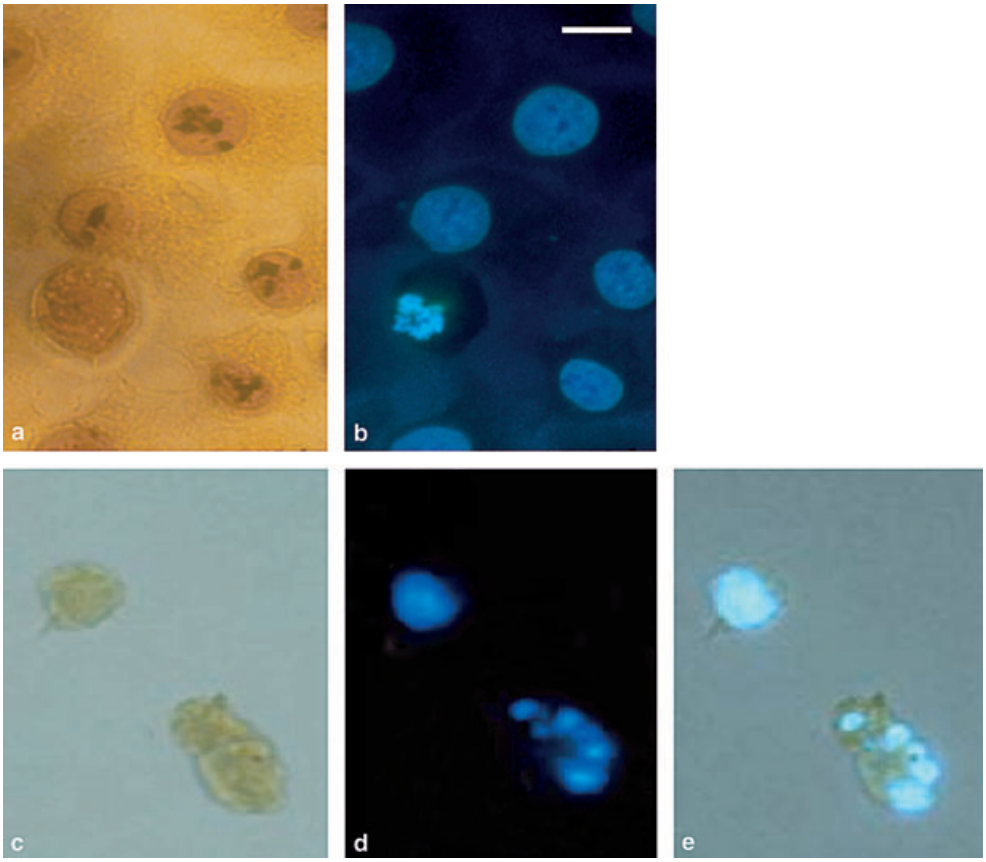

Figure 5 Cytochemical identification of AgNOR proteins in apoptotic human salivary gland (HSG) cells. Apoptosis was induced by treatment with 50 μM cisplatin for 24 h. The staining method is described in the Materials and Methods section. Untreated control cells were stained with $AgNO_3$ (a) and Hoechst 33342 (b). Apoptotic cells were stained with $AgNO_3$ (c) and Hoechst 33342 (d), (e) is a superimposition of (c) and (d). The bar represents 10 μm.

Figure 6 Identification of nucleolin in cellular fractionation in human salivary gland (HSG) cells. The staining method is described in the Materials and Methods section. Whole cell lysates (1 and 2), cytosolic fractions (3 and 4), and nuclear fractions (5 and 6) were prepared from untreated cells (1, 3 and 5) or 50 μM cisplatin for 24 h treated cells (2, 4 and 6). The size of molecular weight is indicated at the right side (kDa).

occurs during mitosis. Those findings suggest that nucleolin might also play an important role in apoptosis. Several studies have examined the role of nucleolin in cell death. Martelli et al. have reported finding a redistribution of nucleolin (associated with fragmentation of nuclei) in apoptotic cells but not necrotic cells (20–22). Brockstedt et al. used two-dimensional electrophoresis to identify nucleolin as the protein that was cleaved in a Burkitt's lymphoma cell line (23).

Consistent with these results, nucleolin might also play an important role in the apoptotic executive phase, a fact confirmed by its cleavage at the time of DNA ladder formation. Activation of protease through unknown mechanisms may cleave the nucleolin, which in turn activates other endonucleases, resulting in DNA ladder formation. Thus, the regulation of the alternation of nucleolin might control one of the apoptotic pathways.

Whether the 95-kDa protein we detected is a cleaved form or a dephosphorylated form of nucleolin is not presently clear. The amount of a 95-kDa protein decreased in MG63 cells treated with okadaic acid (8). The protein phosphatase type 1δ isoform is reportedly associated with nucleolin and might dephosphorylate it (24). These findings suggest that nucleolin could be partially dephosphorylated into a 95-kDa protein during preparation (24, 25). Therefore, we speculated that in apoptosis induced by anticancer drugs, the dynamic alternation of nucleolin begins with 110-kDa nucleolins partially dephosphorylated into 95-kDa proteins while others are cleaved into 80-kDa proteins. Further studies that focus on the appearance of the 95-kDa forms of nucleolin in apoptosis induced by anticancer drugs – in HSG cells are needed.

The results in apoptosis induced by anticancer drugs are a little different to changes in nucleolin that occur in apoptosis induced by okadaic acid. One possible explanation for this contradiction may be different mechanisms of cell injury between anticancer drugs

and protein phosphatase inhibitors. Okadaic acid acts in cytoplasm or on proteins on the cell surface through the Fas/Fas ligand system and induces apoptosis. However, anticancer drugs may directly attack the double- and single-strands of DNA and thus induce cytotoxicity and apoptosis in the cells. Our results differ from those in camptotecin-induced apoptotic HL-60 cells, in which cleavage forms were undetectable (26, 27). This discrepancy may have arisen from using different antibodies rather than from different pathways, different cell lines, or different stages of apoptosis.

Regarding the proteases that cleave 110- and 95-kDa forms of nucleolin into 80-kDa nucleolin, incubation of nucleolin with granzyme A reportedly generates a discrete proteolytic cleavage product of 88 kDa *in vitro* (26). We speculated that the 80-kDa form of nucleolin is a proteolytic fragment of the 110-kDa form of nucleolin cleaved by novel proteases. The proteases involved may be similar to granzyme A (26). Because the apoptosis-inducing granzyme A and interleukin 1β-converting enzyme (ICE) reportedly share at least one substrate, pIL-1 (26), the changes in the nucleolin might be induced by ICE. Direct investigation of the nucleolin structure during apoptosis may be conducted further by purification of nucleolin and immunoblotting with antinucleolin antibodies. Identification of the proteases involved in the cleavage of nucleolin is needed to clarify the executive phase of apoptosis. In addition, further studies are needed to clarify how the fragments of nucleolin are transported from the nucleus to the cytoplasm during apoptosis. However, nucleolin was in cytosolic fraction to the degree of undetectable level, but detectable level in nuclei fraction using cellular fractionation. Therefore, nucleolin should operate as a shuttle protein between the cytoplasm and nucleus (16), but significance of nucleolin's shuttle function in apoptotic alternation remained obscure.

When anticancer drugs were directly mixed with normal cytoplasm and normal nuclei using a cell-free system, DNA ladder formation and alternation of nucleolin were not detectable (data not shown). These findings directly proved that the cleavage of nucleolin might be related to the executive phase of apoptosis, rather than to anticancer drug-induced cleavages. In apoptosis induced by anticancer drugs, protein phosphatase inhibitor (7, 8), or UV irradiation (in preparation), the quantity of the 110-kDa form of nucleolin decreased and the 80-kDa form of nucleolin appeared. These results indicate that alternation in nucleolin and AgNOR proteins occur not only in anticancer drug-induced apoptosis but also in apoptosis induced by other stimuli. We speculate that the cleavages of nucleolin might be universal alternation in the apoptotic cells. The alternations of nucleolin and AgNOR proteins may be clinical indicators of apoptosis. In addition, the regulation of nucleolin cleavage might regulate the apoptotic executive phase and the cytotoxity of anticancer drugs. Additional information along these lines could lead to the future production of a new type of anticancer drugs.

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