Effects of Cesalin on the Ultrastructure and Biological Properties of Cultured Mammalian Cells

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ABSTRACT

Cesalin rapidly inhibits the incorporation of uridine and thymidine into KB, MCF-7, HBL-100, and HTC cells but has no measurable effect on AIAb cells. Protein synthesis is inhibited only after the effect on DNA and RNA is observed. After 12 to 48 hr, the cells in cultures containing cesalin increasingly lose adhesion to the flask surface and float in the medium. Both the inhibition of nucleotide incorporation and the inhibition of the cell growth, used as assays for cytotoxicity, show a varying sensitivity of these cell lines to cesalin, with AIAb cells being the most resistant and KB cells being the most sensitive. The ultrastructural changes induced by cesalin in KB cells demonstrate alteration in the nucleolus, increase in rough endoplasmic reticulum, extensive blebbing of the plasma membrane, and invagination of the nuclear membrane. The blebbing of the plasma membrane decreases after 24 hr with the appearance of a highly disorganized nuclear structure and numerous vacuoles containing insoluble fragments.

INTRODUCTION

Cesalin, an oligomeric protein with a molecular weight of approximately 110,000, is isolated from the endosperm of Caesalpinia gilliesii (9). It exhibits activity in vivo and in vitro against a number of established tumor cell lines (3, 4). Its effects on KB cells have been studied in some detail (4), and in particular it has been noted that (a) respiration is quickly reduced by cesalin without apparent damage to the mitochondria or change in ATP concentrations, (b) the inhibition of protein biosynthesis is preceded by inhibition of DNA and RNA biosynthesis, (c) Na⁺-K⁺-ATPase is partially inhibited, (d) cesalin reacts quickly with the cell surface, and (e) cells cannot be rescued by cesalin antibodies or by repeated washing after exposures of more than 10 to 20 min.

Lectins and polypeptide hormones react with certain cells in a specific manner, leading to profound and particular effects upon the metabolism of the target cells. Since the plasma membranes of tumor cells frequently differ from their normal counterparts and each other, it was of interest to pursue this comparison by extending the evaluation of the effects of cesalin on KB cells to other human cell lines in culture, with particular attention given to the time course of the inhibition of the incorporation of precursors into biopolymers. The use of this inhibition is described as a reliable assay for the quantitation of the biological activity of cesalin.

MATERIALS AND METHODS

Materials. Cesalin was prepared from the endosperm of C. gilliesii as previously described (4, 9). [methyl-³H]Thymidine, [5,6-³H]uridine, and ³H-amino acid mixture were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Cell culture media, serum, and antibiotics were obtained from Grand Island Biological Co., Grand Island, N. Y., except for gentamicin, which was purchased from Microbiological Associates, Inc., Bethesda, Md. All other materials were of reagent grade and from standard sources.

Cells and Tissue Culture Techniques. KB cells (ATCC CCL17), from human oral epidermoid carcinoma, were obtained from the American Type Culture Collection, Rockville, Md. HBL-100 cells (from human milk) and MCF-7 cells (from breast tumor pleural effusions) were obtained from Mason Research Labs, Rockville, Md. AIAb cells (lung metastasis of breast tumor) were originally a gift of Dr. D. Novelli, Oak Ridge Laboratories; during the course of routine passage of the cells, their sensitivity to cesalin changed (4). HTC cells were a gift from Dr. Daryl K. Granner of this department.

KB, AIAb, and MCF-7 cells were maintained as monolayer cultures in Eagle's minimal essential medium with Earle's salts, supplemented with 10% (v/v) heat-inactivated fetal calf serum. HBL-100 cells were maintained as monolayers in N-tris(hydroxymethyl)methylglycine-buffered Swim's S-77 medium with CaCl₂. HTC cells were grown as spinner cultures in Swim's S-77 medium without CaCl₂.

All culture media contained gentamicin at a concentration of 50 µg/ml or penicillin-streptomycin at concentrations of 100 units/ml and 100 µg/ml, respectively. Cells in Earle's minimal essential medium were grown in a 5% CO₂ atmosphere at 37°C. All other cells were grown in closed systems at 37°C. Tests for Mycoplasma contamination throughout this study were negative.

Precursor Incorporation Studies. Cells grown in monolayer were initially seeded at 5 x 10⁵ cells/sq 25-cm Falcon flasks and allowed to adhere and grow for 15 to 18 hr at 37°C prior to the addition of cesalin or PBS. Cesalin dissolved in 100 µl of sterile PBS was added at zero time to a final concentration in the medium of 2 ng/ml. Control cultures received 100 µl of PBS. At the times indicated (Charts 1 to 3), 1.0 µCi of [methyl-³H]thymidine (4.6 Ci/mmol), 2 µCi of [5,6-³H]uridine (40 Ci/mmol), or 2 µCi of ³H-amino acid mixture was added to duplicate cultures. In those flasks receiving labeled amino acids, the medium was replaced with PBS at 37°C prior to addition of the label. The cultures were incubated at 37°C for 10 min for thymidine and amino acids and 20 min for uridine. At the end of this labeling pulse, the medium was aspirated, and the

1 This investigation was supported by Grants GM-550, GM-14013 and CA-17840 from the NIH.
2 This work will form part of a dissertation to the Graduate College of the University of Iowa in partial fulfillment of the requirements for the Ph.D. degree.
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Received June 27, 1979; accepted October 17, 1979.

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monolayers were washed 3 times with ice-cold PBS. For measurement of thymidine and amino acid uptake into the cells and incorporation into biopolymers, the cells were dispersed in 1 ml of 1 N NaOH, and the macromolecules were precipitated after addition of TCA to a final concentration of 15% (v/v). The mixture was centrifuged, and the acid-soluble fraction was counted in a Beckman LS-100C liquid scintillation counter after adding 15 ml of a Triton X-100-xylene-based scintillation fluid. The TCA-precipitated material was dissolved in 0.5 ml of 0.1 N NaOH, neutralized with 50 μl of 1 N HCl, and counted as above. For measurement of uridine incorporation, the cells were removed from the flask with 1.0 ml of Versene solution (0.53 mm) in PBS. The cells were pelleted and washed twice with 1.0 ml of 5% TCA (v/v). The TCA-soluble and -insoluble fractions were counted as before.

Incorporation studies with cells growing in spinner culture were conducted similarly with slight modifications in the initial processing. HTC cells were seeded at 2 × 10^5 cells/ml. After labeling, the cells were pelleted, resuspended in cold PBS, and repelleted after each of 3 PBS washes.

**IDso Determinations.** IDso's were determined by 2 methods: (a) The effect of varying concentrations of cesalin on cell growth was measured by the procedure of Smith et al. (19). Briefly, cells were seeded at 2 × 10^5/4 ml in 16-× 100-mm screw-capped plastic culture tubes. Duplicate cultures were set up for each cesalin concentration and for controls. At the same time the cells were seeded into the tubes, cesalin in 100 μl of PBS or PBS alone was added, the tubes were placed at a 30° angle in a 37° incubator, and the cells were allowed to grow for 3 days. At the end of the 3-day period, the medium was aspirated, and the cells were washed carefully with PBS. The cell protein for control (A controI) and cesalin-treated (A cesalin) cultures was quantitated by the biuret method (5). Protein content was also determined for duplicate tubes at the beginning of the experiment containing the initial number of cells (A0). Duplicate tubes, which were incubated with medium only, were used as a blank. The percentage of inhibition (% I) of control cell growth at each concentration of cesalin was calculated by the following formula:

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\% I = \frac{A_{\text{control}} - A_{\text{cesalin}}}{A_{\text{control}} - A_0} \times 100
\]

(b) The effect of varying concentrations of cesalin on [3H]-thymidine and [3H]uridine incorporation was measured as described above.

**Electron Microscopy.** Cells which had been treated with cesalin (0.5 μg/ml) for the appropriate times were fixed in 1.5% (w/v) glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2. Postfixation was in 1% (w/v) osmium tetroxide and 1.5% K2Fe(CN)6·3H2O in 0.1 M sodium cacodylate, pH 7.2. Cells were dehydrated in acetone and embedded in Spurr’s embedding medium (20), and blocks were sectioned with a Sorvall Porter-Blum MT-2B ultramicrotome. The grid-mounted sections, stained with 5% uranyl acetate followed by lead citrate, were examined in a Hitachi HU-11E electron microscope with an accelerating voltage of 50 kV.

**RESULTS**

Cesalin has been shown to exert a variety of effects on KB cells in culture (3, 4). One of these effects is the inhibition of both thymidine and uridine incorporation after a lag period. In the present work, these inhibition studies were extended to a variety of cell lines, both in monolayer and suspension culture, to compare the relative sensitivities of these cells to cesalin.

The inhibition produced by a constant amount of cesalin (2 ng/ml) on thymidine incorporation in monolayer cultures is most rapid in the KB cells, reaching 20% of control levels by 3 hr (Chart 1). Initially, the HBL-100, HTC, and MCF-7 cells show patterns of inhibition different from those of KB cells, but in each case the incorporation of thymidine has been reduced by 12 hr to 15 to 20% of control values. AIAb cells react very differently in the presence of cesalin, showing an initial slight inhibition that is followed by recovery to control levels at 12 hr. In all cases of monolayer cultures at a cesalin concentration of 2 ng/ml, the cells are still largely attached to the flask surface and exclude trypan blue. Following 24 hr of cesalin treatment, the KB cells become detached from the flask surface and greater than 90% do not exclude vital dyes.

The effect of cesalin on uridine incorporation (Chart 2) is similar to that for thymidine but shows a Faster rate of inhibition. For thymidine and uridine incorporation, respectively, after 3 hr, KB cells show 20 and 10%; MCF-7 cells, 110 and 20%; and HBL-100, 70 and 45% of the respective control cells. Although inhibition of both nucleosides exhibits a lag phase, uridine incorporation is affected as early as after 1 hr and may in fact precede thymidine inhibition. AIAb cells, which show no inhibition of thymidine incorporation with cesalin, have a very slow rate of uptake of exogenous uridine and uracil; therefore, inhibition of RNA synthesis could not be measured by this method.

As was shown previously for KB cells (4), cesalin has a significantly smaller effect on amino acid incorporation in any of the cell lines tested. Not until after 5 hr do inhibition levels reach greater than 50% (Chart 3). It is interesting to note that,
while protein synthesis in AlAb cells is inhibited by cesalin, there does not appear to be an effect on DNA synthesis. A preliminary study indicates that, as in the case of KB cells (4), cesalin does not decrease the soluble pool of nucleotides or amino acids in these cell lines until after significant inhibition of nucleic acid or protein synthesis has taken place.

ID₅₀'s for antitumor agents have usually been measured by the extent of growth inhibition of cells in vivo or in vitro. The in vitro assay used by the National Cancer Institute screening program (17) measures the inhibition of cell growth (defined as protein content) over a 3-day incubation period (see "Materials and Methods"). One major disadvantage of this method is that it will not accurately reflect the biological activity if cell protein is not proportional to cell number, as is the case with macromycin (23). Although the protein:cell or DNA:cell ratios are not disproportionately affected by cesalin treatment up to 24 hr, by this time over 50% of the cells have become detached from the flask surface, and at high concentrations of cesalin all cells become detached by 48 hr. This has the effect of producing an artificially low ID₅₀ because the cesalin-treated cell number is actually less than the initial control count. Since cesalin frequently inhibits both thymidine and uridine incorporation, it was of interest to compare inhibition of these processes with the inhibition of cell protein as a measure of ID₅₀.

All of the monolayer cultures at some concentration of cesalin become detached from the flask surface during the 3-day growth period. KB cells are apparently the most sensitive to this releasing property of cesalin, and concentrations as low as 10⁻⁵ µg/ml cause total release of these cells by 72 hr. This shifts the toxicity curve to lower concentrations of cesalin, resulting in an artificially low ID₅₀ (Chart 4). The inhibitions of both thymidine and uridine incorporation show similar toxicity curves. Contrasted with these data, HTC cells in suspension culture, where growth inhibition is determined directly as a decrease in the number of cells that exclude trypan blue, show similar curves for all 3 methods.

A summary of ID₅₀'s for all the cell lines is shown in Table 1. The values by each method for MCF-7 cells are very close, reflecting the fact that these cells do not become detached as readily as do KB cells. AlAb cells lose adhesion so that again the ID₅₀ of 1.3 x 10⁻³ may be artificially low, whereas these cells show no cesalin sensitivity to inhibition of DNA synthesis. Therefore, the method of ID₅₀ determination by cell protein inhibition for cesalin and macromycin have several disadvantages, and a reasonably quick and more reliable assay can be achieved by measuring the inhibition of incorporation of thymidine or uridine. Similar conclusions have been reported for other antitumor agents (10, 14).

The results from this work and previous studies (2) would indicate that the effect of cesalin on DNA and RNA synthesis cannot be reversed and eventually (24 to 48 hr) causes its
release from the surface and death of the cell. These findings led to a preliminary study of the ultrastructural changes in KB cells that might reflect the nature of the inhibition of cellular syntheses.

The ultrastructure of the KB cell remains similar to that of the control cell (Fig. 1) up to 1 hr after exposure to cesalin. Following 3 hr of cesalin treatment, there is a distinct alteration in the structure of the nucleolus, which has segregated into several components of varying electron density as seen in Fig. 2. This corresponds to a significant inhibition of RNA synthesis by 3 hr as seen in Chart 2. A nearly identical alteration is observed following actinomycin D treatment and seems to be a typical cellular response to inhibitors of RNA synthesis (15).

Following 7 hr of exposure to cesalin, the KB cells demonstrate an increase in the amount of rough endoplasmic reticulum. The nucleoli still retain some variability in density but are becoming more dense and homogeneous than control cells. Further treatment (10 hr) results in a portion of the cells being released from the surface (3). Of the 80% of the total cells that remain attached to the surface after this time, 25% have membranes that show significant zeiotic blebbing (6). Many other attached cells are beginning to bleb (Fig. 3) like those described by Rose (18) and Kessel and Shih (6). The blebs primarily contain particles (which are probably free ribosomes), endoplasmic reticulum, and occasionally other organelles. The cells released from the monolayer are all heavily blebbed (Fig. 5) or contain vacuoles and are electron dense (Fig. 6). As in the adhering cells, the blebs contain primarily free particles, but also the majority of the endoplasmic reticulum. The nucleolus is no longer present as an individual structural unit and the nucleolus contains a significant amount of highly condensed material. In many of the blebbed cells, the nuclear membrane is invaginated. The other morphological type present in the released cells (Fig. 6) contains a densely staining cytoplasm with elliptical cytoplasmic vacuoles beginning to appear, some of which appear to contain sheets of membranes.

All of the treated KB cells have been released from the monolayer after 24 hr and the majority, being dead or dying, no longer exclude trypan blue. As opposed to the released cells at 10 hr when the majority of the cells are blebbed, only a small percentage of the cells released at 24 hr are blebbed. The cytoplasm in most cells is granular in appearance, the endoplasmic reticulum is swollen, and the nucleus is highly invaginated and disrupted, making it difficult to follow the continuity of the nuclear envelope (Fig. 4). Frequently, the nuclear membrane has swollen, presenting a large gap between the 2 layers. The cytoplasmic side of the membrane contains many attached particles, with the space in the nuclear membrane containing a lightly staining flocculent material.

**DISCUSSION**

The inhibition of biopolymer synthesis and the ultrastructural effects produced by cesalin suggest its classification among those antitumor agents that primarily inhibit RNA synthesis (8). Only one other antitumor protein, globimycin, is thus classified; globimycin inhibits both the initiation and termination of RNA synthesis (1). Although cesalin eventually inhibits DNA synthesis, the rate is slower than that seen for RNA, unlike the antitumor proteins macromomycin, neocarzinostatin, and sporamycin (8), from which cesalin also differs in not cleaving existing DNA (22).

Cesalin has been shown to prolong the life of mice bearing Sarcoma 180 or rats with Walker 256 carcinoma (21) and is not toxic to these animals at concentrations that inhibit tumor cell growth. In the present study, HBL-100, a normal human mammary cell line, was somewhat more resistant to cesalin than was the mammary tumor cell MCF-7; the AIAb mammary tumor cell line was the most resistant. Preliminary data suggest that this is the result of fewer cesalin receptors on AIAb cells than on KB cells, the most sensitive cell line.

The observed ultrastructural and biochemical changes in nucleic acid and protein synthesis induced by cesalin present a series of events that ultimately result in cell death. The nucleolar segregation observed early after cesalin treatment, along with the inhibition of uridine incorporation, lends support to the primary effect on RNA synthesis. The concomitant inhibition of oxygen metabolism and Na⁺-K⁺-ATPase activity (3, 4) is not reflected in any obvious early structural changes in the mitochondria or plasma membrane or alterations in intracellular nucleotide pools (4). However, after cesalin treatment of KB cells for 10 hr, the initial blebbing of the plasma membrane and the progressive dilation of both the endoplasmic reticulum and the nuclear envelope suggest a substantial modification in the electrolyte balance by either modification of the integrity of the membrane, alteration of transport systems, or indirectly through interruption of cellular respiration and glycolysis (2). The early proliferation of endoplasmic reticulum and the active period of zeiotic blebbing may also be related to a cellular attempt to overcome the effects of the drug through metabolic processes or mechanical isolation. Blebbing has been observed to occur naturally in several cell lines, where it appeared to be linked to the G₁ phase of the cell cycle (16). There is no suggestion that cesalin blocks the cells in G₁ (4). In the initial stages of cesalin toxicity, the cells completely detach from the monolayer. The nuclear structure is highly disorganized and its contents are aggregated. At this point, the nuclear membrane has begun to swell, possibly due to the build-up of a proteinaceous matrix between the membrane layers. The ultimate result of cesalin treatment is the alteration of nuclear structure to such a degree that the cell cannot recover.

The ultrastructural effects resulting from cesalin treatment are clearly different than those produced by the antitumor protein macromomycin (23). While cesalin causes the swelling of cellular membranes, macromomycin produces an increase in cell size followed by cell lysis. Also, the increase in lipid droplets and general proliferation of organelles produced by macromomycin is absent in cesalin-treated cells. Light microscopy revealed that similar morphological effects were observed in the cells treated with cesalin and the antitumor protein flamulin (7); these changes remain to be characterized at a higher resolution.

Studies are currently under way to determine the fate of cesalin after binding to the cell surface⁶ and the nature of its cellular receptor. Unlike the toxic proteins abrin (13), ricin (12), and modeccin (11), which are also isolated from plant sources, cesalin is not a lectin; preliminary evidence points to the cesalin receptor as being a proteolipid (8).

**ACKNOWLEDGMENTS**

We wish to thank Kenneth C. Moore for assistance and consultation with the

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⁶ V. L. Shepherd and R. Montgomery, unpublished work.
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Electron microscopic studies, Susan J. Taylor for technical assistance in the tissue culture studies, and Jane Mead for the preparation of cesalin.

REFERENCES

Fig. 1. Typical KB cell from control cultures. x 7700.

Fig. 2. KB cell showing the altered nucleolar structure and increase in the rough endoplasmic reticulum after 3 hr of cesalin treatment. x 5100.

Fig. 3. Following 10 hr of cesalin treatment, the KB cells remaining attached to the monolayer begin to show the appearance of peripheral surface blebbing and membrane herniations (arrows). x 7100.

Fig. 4. KB cells showing a highly blebbed and electron-dense nucleus. A significant number of swollen cytoplasmic membranes are also present and appear to be the remains of the rough endoplasmic reticulum and nuclear envelope. x 7100.
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Fig. 5. A KB cell released from the monolayer showing extensive zelliot blebbing and condensation of nuclear components. The cell had been exposed to cesalin for 10 hr. × 9300.

Fig. 6. A KB cell released from the monolayer after 10 hr of cesalin treatment. The cytoplasm contains many electron-dense bodies and is filling with small vacuoles, the larger of which contain membrane fragments. × 9300.
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