# A Note on the Decreased Number and Loss of Fibrillar Centres in Nucleoli of Apoptotic HL-60 Leukaemic Granulocytic Precursors Produced by 5-Aminolaevulinic Acid-Based Photodynamic Treatment*

( 5-aminolaevulinic acid / photodynamic effect / HL-60 cells / nucleolar fibrillar centres / apoptosis)

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**Abstract. The nuclear and nucleolar ultrastructure was studied by means of conventional transmission electron microscopy to provide more and complementary information on nucleolar changes accompanying the apoptotic process in leukaemic granulocytic precursors (HL-60 cells) produced by PDT without previous terminal differentiation. PDT induced the apoptotic process using BL irradiation and ALA as a precursor of the photosensitizer protoporphyrin IX. PDT produced marked changes of the nucleolar ultrastructure in apoptotic cells, such as reduction of the number and loss of fibrillar centres surrounding dense fibrillar components. Such nucleolar changes are known to reflect an alteration of nucleolar biosynthetic activities, which are believed to be located at the periphery of fibrillar centres. Some electron micrographs also indicated that fibrillar centres apparently migrated out from nucleolar bodies.**

Recent light microscopic studies demonstrated that the photodynamic treatment (PDT) using 5-aminolaevulinic acid (ALA) as a precursor of the photosenzitizer protoporphyrin IX and blue light (BL) irradiation produced the apoptotic process without preceding terminal differentiation in leukaemic granulocytic precursors – HL-60. After PDT, HL-60 cells exhibited marked light microscopic nucleolar alterations, which accompanied nuclear changes characteristic for the apoptotic process (Smetana et al., 2000). Such nucleolar alterations were mainly represented by a reduction or disappearance of the interphasic silver-stained nucleolus organizer regions (AgNORs) which, as generally accepted, reflect the decrease or inhibition of the nucleolar biosynthetic and cell proliferation activities (Busch et al., 1979; Trere et al., 1989). Therefore, the present study was undertaken to provide more information on PDT-induced nucleolar alterations and mainly on fibrillar centres in apoptotic cells at the electron microscopic level. The results demonstrated that such treatment produced a significant reduction and disappearance of fibrillar centres in compact nucleoli, which was also accompanied by the reduction of surrounding dense fibrillar components. Such changes of the nucleolar ultrastructure, similarly as light microscopy (Smetana et al., 2000; Morimoto et al., 2001), also reflected the altered nucleolar functions in individual apoptotic cells.

## Material and Methods

### *Photodynamic experiments*

The cells (cell density 1.5 x $10^5$/ml) were incubated with 1 mM ALA in RPMI 1640 medium supplemented with 25 mM HEPES and 10% FCS (Sigma-Aldrich, St. Louis, MO) in 25 $cm^2$ culture flasks. Then, freshly prepared 100 mM ALA solution in RPMI 1640 medium was added to a final concentration of 1 mM (167 μg/ ml). The cells were incubated at 37˚C for 4 h, resuspended in a fresh medium (not containing phenol red), supplemented with 10% FCS and exposed for 1 h to broad-spectrum BL (two Osram Dulux DS9/71 discharge lamps of 2.3 W radiant power in the 400–550 nm range) from 25 cm distance. In that way a light dose of 18 J/$cm^2$ was delivered to the cells. Subsequently, the cells were incubated for 1 h at room temperature (in the dark) and the cell proliferation was assessed by flow cytometry-bromodeoxyuridine (BrdU) incorporation (Grebeňová et al., 1998). The fraction of apoptotic cells was deter-

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*Dedicated to Prof. H. Busch on the occasion of his 80th birthday

Abbreviations: ALA – 5-aminolaevulinic acid, AgNORs – silver-stained nucleolus organizer regions, BL – blue light, BrdU – bromodeoxyuridine, PDT – photodynamic treatment.

mined by flow cytometry employing the terminal deoxynucleotidyl transferase nick end labelling (TUNEL) method using an APO-BRDU labelling kit (Phoenix Flow Systems, Inc, San Diego, CA) following the manufacturer's protocol. In short: 1 x $10^6$ cells were fixed with paraformaldehyde solution (5 ml of 1% w/v), pelleted by centrifugation and resuspended in ice-cold 70% (v/v) ethanol. The DNA 3'OH ends were labelled for 60 min with BrdU under catalysis of the terminal deoxynucleotidyl transferase (TdT) enzyme. The BrdU-labelled 3'OH terminals were detected by flow cytometry using fluorescein-labelled anti-BrdU antibody monitored at 520 nm. Untreated HL-60 cells served as controls and three separate experiments were performed.

### *Electron and light microscopy*

Samples of HL-60 cells were prepared by centrifugation after one-hour incubation and treatment under conditions described above. The resulting pellets were fixed in l.6% glutaraldehyde or 2% osmium tetroxide in phosphate buffer, pH 7.2, for 1 h at room temperature. Then, specimens postfixed and dehydrated in graded ethanols containing uranyl acetate were embedded in Durcupan (Fluka, Buchs, Switzerland) replacing Araldite in the mixture with Epon (Serva, Heidelberg, Germany) according to Mollenhauer (Mollenhauer, 1964; Smetana, 1970). Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with Philips 300 and Philips Morgagni 268 electron microscopes. Electron microscopic images were captured with digital imaging software of the Morgagni 268 electron microscope, further processed and printed using the Microsoft Power Point Program and a Hewlet-Packard Deskjet 930C printer.

For the light microscopic control of the incidence of apoptotic cells and bodies, the percentage of cells with a condensed chromatin structure characteristic for the apoptotic process as well as the percentage of apoptotic bodies were determined in specimens prepared by cytocentrifugation using a Shandon II centrifuge (see Smetana et al., 2000).

## Results

### *The incidence of proliferating and apoptotic cells or bodies (Table 1)*

Almost all control cells incorporated BrdU as measured by flow cytometry and mitotic divisions were occasionally seen in cytospin preparations. In contrast, PDT with ALA and BL substantially reduced the percentage of cells incorporating BrdU as measured by flow cytometry, and mitotic divisions were absent in cytospin preparations. The incidence of apoptotic cells and bodies was very small in controls. PDT significantly increased the percentage of apoptotic cells and bodies regardless of the method used for their detection.

### *Electron microscopy*

In control specimens, the structural organization of nucleoli did not differ from previous descriptions of compact nucleoli with less distinct nucleolonemas (Busch and Smetana, 1970; Schwarzacher and Wachtler, 1991; Wachtler and Stahl, 1993). Such nucleoli contained several distinct fibrillar centres (Table 1, Fig. 1), which were surrounded by dense fibrillar components (Fig. 2). The rest of the nucleolar body possessed dense granular components (Figs. 1, 2).

In contrast to control cells, PDT with ALA and BL in apoptotic cells substantially reduced the number of fibrillar centres surrounded by dense fibrillar components (Table 1). Such nucleoli in apoptotic cells with characteristic chromatin condensation appeared very compact and some of them did not contain any fibrillar centre (Figs. 3, 4). Regions containing dense fibrillar components were practically not visible in such nucleoli and, therefore, dense granular components were very prominent (Figs. 3, 4). On the other hand, it seems to be interesting that compact nucleoli without distinct fibrillar centres were also present in nuclei without chromatin condensation characteristic for apoptotic cells (Fig. 5). However, such cells occasionally exhibited cytoplasmic large buds resembling blebbing (Fig. 5), which is considered to be one of early signs of the cell death and apoptotic process (Bessis, 1973; Willingham, 1999). A few compact nucleoli in cells without chromatin condensation also possessed fibrillar centres at the nucleolar periphery, suggesting their extrusion from the nucleolar body (Fig. 6). Thus, a possibility exists that such phenomenon might reflect the nucleolar transformation to compact nucleoli without fibrillar centres.

*Table 1. The effect of ALA-induced PDT on the incidence of apoptotic cells and bodies, BrdU incorporation and number of nucleolar fibrillar centres*

| **Treatment** | Apo[a] | AC+AB[a] | BrdU[a] | **FC**[b] |
|---|---|---|---|---|
| 0 | **4.3** (1.0) | **11.1** (0.7) | **100.0** | **3.5** (0.4) |
| ALA+BL | **58.8** (1.4) * | **58.9** (3.2) * | **39.5** (4.6) * | **1.1** (0.3)* |

[a] percentage, **mean** and standard deviation

[b] **mean** and standard deviation

* significant difference in comparison with controls using the t-test ($P < 0.001$)

0 – control untreated cells

Apo – apoptotic cells - TUNEL assay - flow cytometry

AC+AB – apoptotic cells with characteristic chromatin condensation and apoptotic bodies in cytospin preparations evaluated by means of light microscopy

BL – broad spectrum of blue light

BrdU – incorporation determined after incubation with BrdU labelling solution for 18 h

FCs – number of fibrillar centres (evaluated in 100 nucleolar profiles larger than 1 μm)

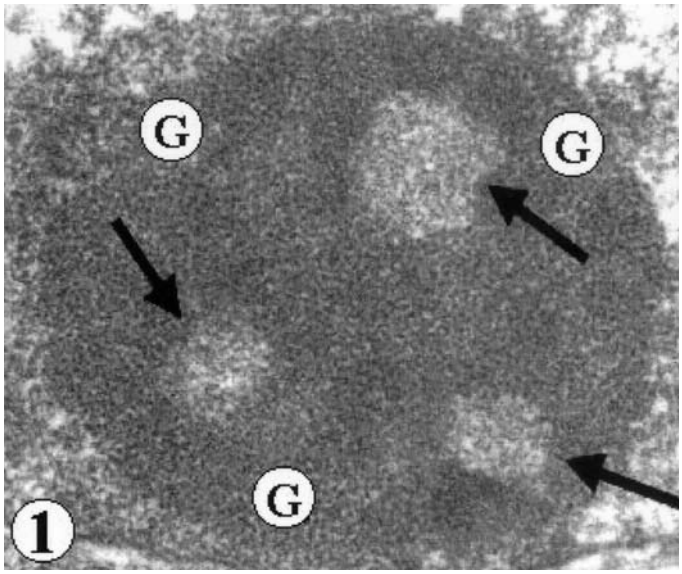


*Fig. 1.* A nucleolus of a control cell contains three distinct fibrillar centres (arrows) surrounded by fibrillar components. Granular components (G) are in the nucleolar peripheral part. Magnification approx. 28,000x.

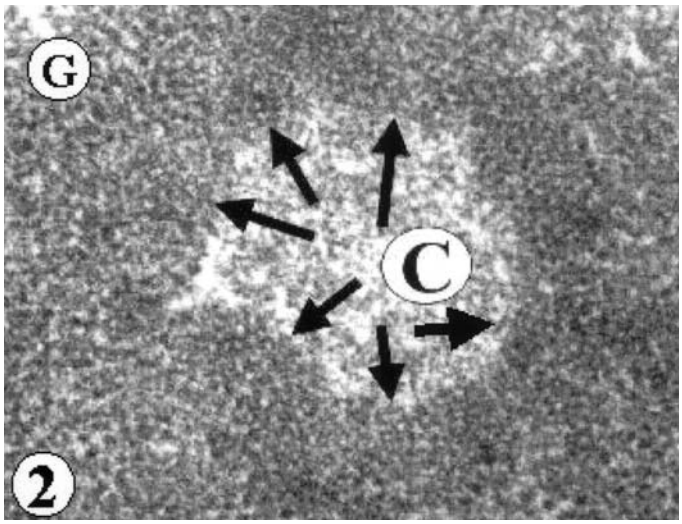


*Fig. 2.* A nucleolus of a control cell. A fibrillar centre (C) is surrounded by dense fibrillar components (arrows). Granular components – G. Magnification approx. 80,000x.

## Discussion

The presented data provided a complementary and new information on nucleolar changes produced by PDT in individual apoptotic cells. It was not surprising that nucleolar fibrillar centres in apoptotic cells were decreased in number or disappeared after that treatment. Such observations are in harmony with the previous cytochemical study of this laboratory, which demonstrated the reduction and disappearance of AgNORs in these cells (Smetana et al., 2000). On this occasion it should be mentioned that AgNORs visualized by light microscopy as silver-stained particles correspond to fibrillar centres surrounded by dense fibrillar components in the electron microscope (Schwarzacher and Wachtler, 1983; Lafarga et al., 1995; Smetana et al., 1995, 1999). Thus, both the present electron microscopic and previous light microscopic observations on PDT reflect the decrease or inhibition of the nucleolar biosynthetic activities in individual cells, which take place in nucleolar regions adjacent to fibrillar centres (Raška and Dundr, 1993; Wachtler and Stahl, 1993; Hozák et al., 1994; Smetana et al., 1995). The decreased incidence or disappearance of dense fibrillar components in compact nucleoli of apoptotic cells observed in the present study are also in harmony with this interpretation. Nucleolar dense fibrillar components are known to contain newly transcribed ribosomal RNA (Busch and Smetana, 1970; Schwarzacher and Wachtler, 1991; Raška and Dundr, 1993; Wachtler and Stahl, 1993; Hozák et al., 1994).

The large amount of granular RNP components and thus the granular appearance of compact nucleoli in

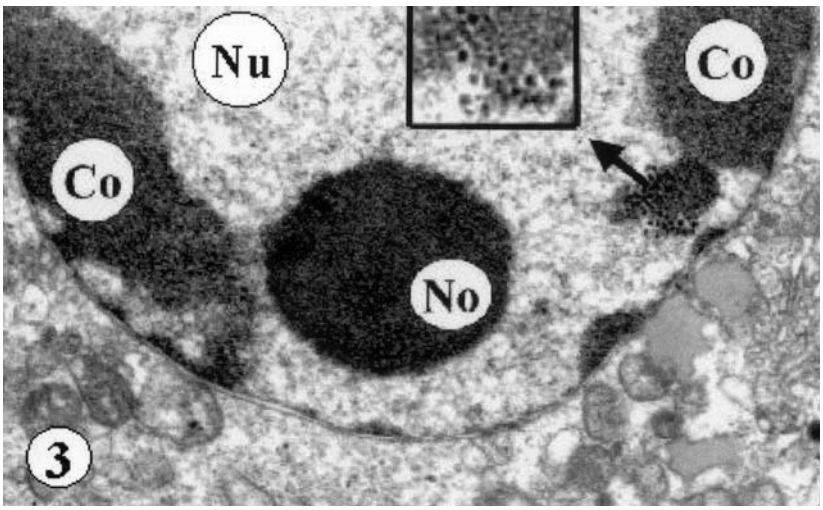


*Fig. 3.* PDT. A compact nucleolus without fibrillar centres (No). The nucleus (Nu) is characteristic for the apoptotic process because of a heavy chromatin condensation (Co) at the nuclear membrane and clumping of perichromatin granules (arrow and insert). Nu – Nucleus. Magnification approx. 15,500x and 35,000x (insert).

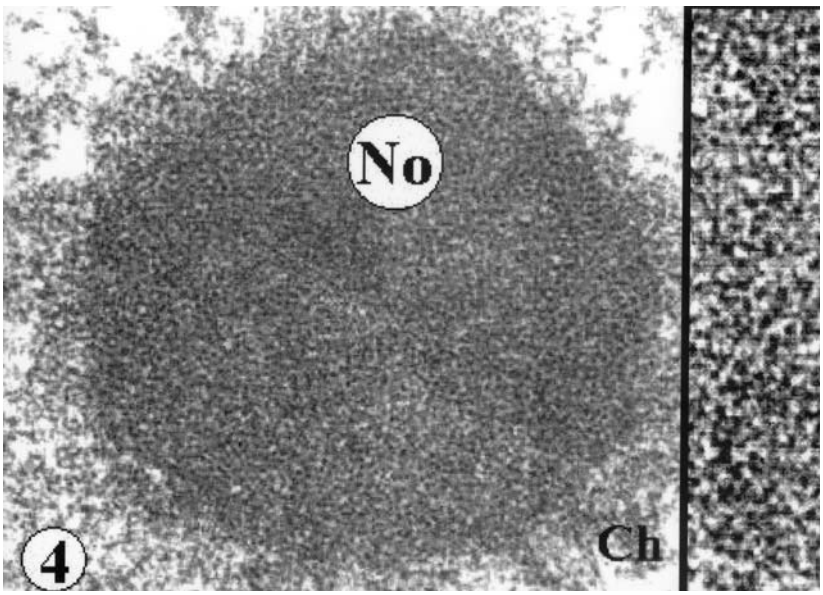


*Fig. 4.* PDT. A compact nucleolus (No) without fibrillar centres and with distinct granular RNP components, which are magnified in the insert. Chromatin surrounding the nucleolar body – Ch. Magnification approx. 31,000x and 75,000x (insert).

apoptotic cells accompanied by the disappearance of dense fibrillar components might be produced by the conversion of dense fibrillar to dense granular components without renewal of the former, as mentioned above. Dense fibrillar components containing the newly transcribed rRNA are precursors of granular RNP components in which this nucleic acid is, at least partially, processed (Raška and Dundr, 1993; Wachtler and Stahl, 1993; Staněk et al., 2000).

In addition, the reduced number or loss of AgNORs (Smetana et al., 2000) and fibrillar centres (present study) just illustrates the abolished proliferating capacity of individual apoptotic cells, because these nucleolar components are considered to be proliferation markers (Trere et al., 1989). Moreover, the reduced incidence of cells incorporating BrdU and the absence of mitotic division after PDT is also in harmony with such conclusion.

The presence of a fibrillar centre in the nucleolar periphery sticking out from the nucleolar body suggests how nucleoli might lose this nucleolar compartment. The extrusion of fibrillar centres from nucleoli was already observed in earlier studies on a variety of cells, but unfortunately without any explanation of this phenomenon (Smetana et al., 1971; Bouteille et al., 1974). Thus, a possibility also exists that some simple nuclear bodies might originate from extruded fibrillar centres.

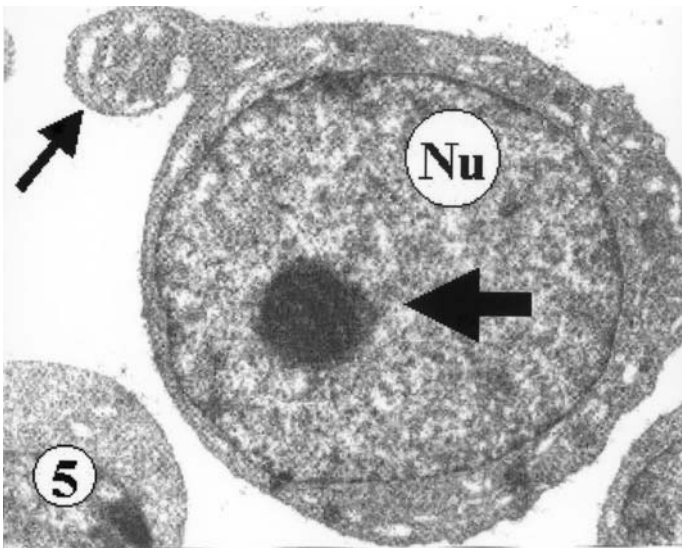


*Fig. 5.* PDT. A compact nucleolus (large arrow) without distinct fibrillar centres. Note that the cell is characterized by a narrow rim of the cytoplasm, which exhibits a large bud (small arrow) However, the nucleus (Nu) does not exhibit a chromatin condensation characteristic for an apoptotic cell. Magnification approx. 8,000x.

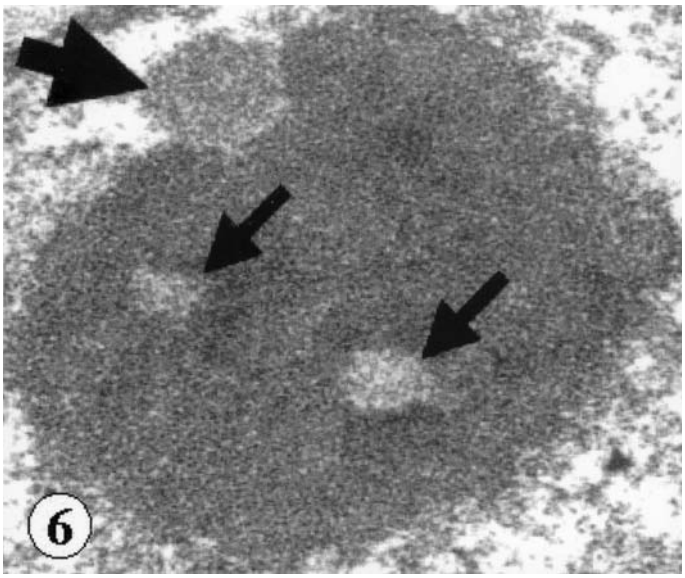


*Fig. 6.* PDT. The nucleolus contains three distinct fibrillar centres (arrows). Note that one of them protrudes out of the nucleolus (short thick arrow). Magnification approx. 34,000x.

At the end of the discussion it should be mentioned that the presented observations also contributed to the previously known variability of nuclear and particularly nucleolar structural changes in apoptotic cells. However, all presently and previously observed nucleolar changes in aging or apoptotic cells always indicated the disturbed nucleolar function, which apparently might contribute to the apoptotic process (Biggiogera et al., 1998, 2000; Martelli et al., 2001).

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