Simultaneous Detection of DAB and Methyl Green Signals on Apoptotic Nuclei by Confocal Laser Scanning Microscopy

Johbu Itoh¹, Shinobu Umemura², Hideaki Hasegawa¹, Masanori Yasuda², Susumu Takekoshi¹, Yoshiyuki R. Osamura² and Keiichi Watanabe²

¹Laboratories of Structure and Function Research, ²Department of Pathology, Tokai University School of Medicine, Isehara, Kanagawa 259–1193, Japan

Received June 4, 2003; accepted June 23, 2003

Observations of the fine structures of subcellular organelles and their pathophysiological changes have been made by many investigators by utilizing the many advantageous functions of confocal laser scanning microscopy (CLSM). However, reports of CLSM observations of fine inner structural changes of nuclei are rather rare.

The usefulness and value of counterstaining in histochemical staining is widely appreciated. While observing methyl green (MG) nuclear-counterstained sections by CLSM, we found that fluorescence emitted through MG was clearly detected by CLSM. It is generally accepted that MG reacts specifically to double stranded (dbs)-DNA, as was proved by Umemura [30] in our laboratory.

Key words: methyl green-fluorescence, DAB, TUNEL, rat small intestine, CLSM

I. Introduction

We have attempted to evaluate structures at the subcellular level in light microscopic specimens. This approach is particularly important in the fields of immunohistochemistry and enzyme histochemistry because these methods localize particular substances in subcellular organelles. For these purposes, various approaches have been attempted [15, 20]. Laser scanning microscopy has been utilized as CSLM using fluorescence [15, 23]. Recently, the application of CSLM combined with computer image analysis in the field of cell biology has been expanded. CSLM now enables one to observe the subcellular localization of various biologically active substances utilizing routinely processed light microscopic specimens [1–3, 8, 13, 16, 26, 33].

Methyl green (MG) has widely been employed for nuclear counterstaining in various histochemical methods for the following reasons [3, 4, 24]. 1. Due to the validity of its use in histochemistry (immuno-, enzyme histochemistry and other modified methods), MG has widely been employed for nuclear counterstaining in various histochemical methods (bright field light microscopic observations). 2. MG staining is very simple and easy, and provides excellent contrast to most histochemical reaction products in terms of color. In a monochrome photograph, MG does hinder the view of histochemical reaction products (the greenish color can easily be eliminated with a filter).

MG has been known to stain double stranded DNA (Pearse 1985). While Pearse reported that MG has affinity for intact or double-stranded DNA, other researches sometimes reported a decrease of MG staining on heating sections for antigen retrieval [5, 10, 11, 17, 19–21, 25, 27, 28, 30, 31].
The phenomenon suggests that MG staining is influenced by structural alteration of the DNA chain.

Umemura et al. reported MG staining mechanisms [30].

In this report, MG-stained nuclei of the absorptive cells (terminal differentiating cells) of rat intestinal villi were observed by CLSM, and the changes in intensity and distribution of MG staining were examined. The cells were also identified as slowly undergoing apoptosis [9]. The apoptosis is thought to occur during the process of terminal differentiation, which takes about 48 hr [5, 6, 9, 17, 18]. To examine those apoptotic changes, a TUNEL method in which the 3’ends of fragmented dsb-DNAs are labeled with histochemically detectable dUTP was employed [7, 25, 28, 29, 32, 33].

II. Materials and Methods

Experimental animals and tissues

Wistar Imamichi rats, aged 10 weeks and weighing approximately 300 g, purchased from the Imamichi Institute for Animal Reproduction (Tokyo, Japan), were used in the experiments. These rats were anesthetized with diethyl ether and then perfused via a cannula inserted into the aorta with 0.03 M PIPES buffer. This process was followed immediately by fixation with 4% parafomaldehyde (PFA) in PIPES for 5 min, after which the small intestines were excised. Small intestines, poptiloid and/or mesenterial lymph nodes were excised and subjected to various forms of histochemical staining with MG counterstaining. In untreated rat small intestines, absorptive epithelial cells at various stages maturation were stained by the TUNEL method to detect apoptotic cells. Tissue blocks fixed with 4% PFA for 12 hr were embedded in paraffin. These paraffin blocks were cut into 4-μm-thick sections which were then adhered to 3-aminopropyl-trimethoxysilane coated-glass slides (Superfrost S8443; Matsunami Glass, Osaka, Japan). After deparaffinization, sections were treated with proteinase K (Sigma Chemical, St Louis, MO) in PBS (20 g/ml) for 15 min at RT. After the sections had been washed with PBS, endogenous peroxidase activity was blocked using 0.3% H2O2 in methanol for 30 min at RT. TdT and biotin-16-dUTP were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). The TUNEL procedure employed was described previously in detail [6]. Sections were stained with 0.025% 3-3’-diaminobenzidine tetrahydrochloride (DAB) in Tris-HCl buffer and counterstained with 5% MG, which was processed with purification by chloroform. As a negative control, sections were incubated in the absence of TdT.

Results of the TUNEL method to detect apoptotic cell-positive reaction products and fluorescence emitted through MG were compared.

Confocal laser scanning microscopy (CLSM)

CLSM (LSM-410, Carl Zeiss, Jena, Germany) was employed on paraffin sections to elucidate the relation between MG-stained nuclei and the TUNEL signals based on the color intensity profile, projection. MG signals were detected with excitation at 543 nm using a helium neon laser, which emitted a wavelength of 570–590 nm.

Non-fluorescent as well as fluorescent signals are detectable by CLSM [11–14, 16, 22, 26]. The non-fluorescent signals including HRP-DAB-OsO4, osmium black, azo dye, nitroblue tetrazolium (NBT), and heavy metals have advantages over fluorescent signals in that they are permanently preserved and allow repeated or retrospective examination [11, 13, 14, 16]. For example, a 543-nm wavelength helium-neon laser ray illuminated DAB-OsO4 reaction products. Optical reflectance signals from DAB-OsO4 reaction products were observed using a dichroic beam splitter (NT 80/20/543, Carl Zeiss, Jena, Germany) without an emission filter. Double- or triple-labeled volumetric data sets of these images taken with a confocal laser scanning microscope can be digitized and made suitable to image analysis manipulation and computer-assisted 3D reconstructions using LSM software version 3.98 (Carl Zeiss, Jena, Germany). Their nuclei were visualized with a C-apochro-mat (×63, water, N.A. 1.25, Carl Zeiss) and a Plan-Neofluar (10×, N.A. 0.30; 20×, N.A. 0.50, Carl Zeiss) objective lens, and a stepping motor unit for axial scanning at focus steps of 0.5 μm. The image resolution was 512×512 and/or 1024×1024 pixels (8 bit, 256 gray levels).

TUNEL method

Paraffin blocks were cut into 4-μm-thick sections and adhered to 3-aminopropyl-trimethoxysilane coated-glass slides (Superfrost S8443; Matsunami Glass, Osaka, Japan). After deparaffinization, sections were treated with proteinase K (Sigma Chemical, St Louis, MO) in PBS (20 μg/ml) for 15 min at RT. After the sections had been washed with PBS, endogenous peroxidase activity was blocked using 0.3% H2O2 in methanol for 30 min at RT. TdT and biotin-16-dUTP were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). The TUNEL procedure employed was described previously in detail [6]. Sections were stained with 0.025% DAB in Tris-HCl buffer and counterstained with 5% MG, which was processed with purification by chloroform. As a negative control, sections were incubated in the absence of TdT.

TUNEL-stained paraffin sections were used for subcellular observation by CLSM. To enhance the TUNEL signals visualized by DAB, which were identified in the reflection mode, sections were reacted with 0.2% OsO4 in cacodylate buffer for 1 hr.

Membrane analysis

The protocol was briefly described as follows; Single stranded (ss) DNA was prepared by denaturing salmon sperm double stranded (ds) DNA with heating at 100°C for 5 min, or dsDNA was prepared by hybridization of an oligo-probe, ssDNA and Nicked DNA. For protocol particulars, refer to Umemura et al. [30].
III. Results

While observing MG-counterstained section by CLSM, we noticed that chromatin distribution patterns were clearly visible using the fluorescence emitted through MG (Fig. 1) [24]. The excitation was at 543 nm with a helium neon laser, which emitted a wavelength of 570–590 nm. Hematoxyline which is most popularly used for counterstaining failed to emit such fluorescence. Actually, the fluorescence generated by MG in CLSM was clearly detectable in the present experiment. The fluorescent intensity of MG in each DNA was detected by CLSM. The fluorescence intensity of MG decreased in the order of a, c, d, and b (Fig. 1).

Localization of MG to nuclei of absorptive epithelial cells of rat small intestine

The intestinal villi were divided into three parts, cells in the crypt (Figs. 2d, 3d, 4d, 5d), cells in the midportion or midzone (Figs. 2c, 3c, 4c, 5c) and cells in the tips (Figs. 2b, 3b, 4b, 5b). Histochemical observation by conventional light microscopy (Fig. 2) revealed the nuclei of absorptive cells in intestinal villi. Fig. 2a gives a general view of intestinal villi.

![Diagram showing fluorescence intensity of MG in each type of DNA as determined by CLSM. a: double-strand DNA. b: single-strand DNA. c: annealing DNA. d: nick-DNA. Fluorescent intensity: a>c>d>b. The result indicated that MG reacts predominantly with double-stranded DNA. I: Dilution x2, II: Dilution x4, III: Dilution x6.](image)

**Fig. 1.** MG is most widely employed for nuclear counterstaining in various histochemical methods (bright field light microscopic observations). Fluorescent intensity of MG in each type of DNA as determined by CLSM. a: double-strand DNA. b: single-strand DNA. c: annealing DNA. d: nick-DNA. Fluorescent intensity: a>c>d>b. The result indicated that MG reacts predominantly with double-stranded DNA. I: Dilution x2, II: Dilution x4, III: Dilution x6.
The nuclei of cells in the crypt are stained slightly more intensely than those in other parts. Cells in the tips are stained weakly (Fig. 2b); however, the intranuclear distribution of MG staining can be observed. Histochemical observation
Simultaneous Detection of DAB and Methyl Green

Fig. 3. Validity of MG counterstaining in CLSM. Histochemical staining of MG in untreated rat small intestine by CLSM. This figure exhibits fluorescence due to MG in CLSM. **a:** general view of intestinal villi. **d:** right side shows nuclei of crypt cells. The most intense fluorescence was noted in these cells (compared with nuclei of cells in other parts, for instance, the midportion cells and tip cells.) **c:** nuclei of midportion or midzone cells. **b:** nuclei of cells in the tip of villi. Nuclei of crypt cells exhibited the most intense fluorescence. In these nuclei MG distributed diffusely. Mid portion cell nuclei show weak fluorescence. Note the fluorescence in the periphery of the nucleus and nucleoli. The fluorescence is far more intense than that in other parts of the nucleus. In the nuclei of tip cells which are shown in **b,** this tendency was more conspicuous. That is, only weak fluorescence was exhibited at the periphery of the nuclei which corresponds to heterochromatin. Bars=100 μm (**a**), 5 μm (**b–d**).
and thickly. Midportion cell nuclei showed weak fluorescence (Fig. 3c). It is worth noting that fluorescence was detected in the periphery of nuclei and nucleoli. The fluorescence was far more intense than that in other parts of the nucleus. In the nuclei of tip cells which are shown in Fig. 3b, this tendency became more conspicuous. That is, only weak fluorescence existed at the periphery of the nucleus which corresponds to heterochromatin.

In the course of terminal functional differentiation, newly generated absorptive cells in the crypt reach the tip of the villi in 2 days. It is generally accepted that those cells in the tip of villi undergo apoptotic cell death [9].

We performed TUNEL experiments on the small intestine (Fig. 4). Positive staining appears as brownish deposits and the nuclei are stained green by MG. Cells in the crypt do not show any positive TUNEL staining (Fig. 4d). Nuclei in the midportion cells show weak staining (Fig. 4c). The staining clearly intensified in the nuclei of tip cells but the intranuclear distribution of the staining remained obscure.

Fig. 4. TUNEL analysis of untreated rat small intestine by LM. Positive TUNEL staining exhibits as brownish deposits and nuclei are stained green by MG. d: cells in the crypt do not show any positive TUNEL staining. c: nuclei in midportion cells show weak TUNEL staining. The staining clearly intensified in the nuclei of tip cells but the intranuclear distribution of the staining remained obscure.
Simultaneous Detection of DAB and Methyl Green

The intranuclear distribution of the positive staining remained obscure (Fig. 4b).

The same specimens were observed under a confocal laser scanning microscope (Fig. 5). The signals from MG were detected in the fluorescence mode and exhibited a greenish color. Signals from TUNEL-stained deposits, which were actually the DAB-osmium compound, were detected by reflectance mode CLSM and exhibited a reddish color. The signals obtained in the CLSM were overlaid using a computed image analysis system and the images produced are shown in this figure. Colocalization sites of MG and TUNEL reaction products are exhibited in orange, a mixture of green and red. Nuclei in the crypt cells are shown in d. The entire nucleus is diffusely covered in greenish fluorescence and TUNEL reaction products appearing as reddish deposit are almost negligible. c: nuclei of mid zone cells. Greenish fluorescence is markedly diminished and shifted to the nuclear periphery which may correspond to heterochromatin. Orange deposits which were based on TUNEL reaction products also localized in the nuclear periphery. b: nuclei of tip cells. Orange deposits in the nuclear periphery markedly increased compared with those of midzone cells. A disproportionate decrease of greenish fluorescence was also evident. Bars=100 μm (a), 5 μm (b–d).
Itoh et al.
374

Actually DAB-osmium compound, were detected in the reflection mode by CLSM and exhibited a reddish color (Figs. 5, 6a). The two signals were then merged using a computed image analysis system. The images produced are shown in Figs. 5, 6a. Colocalization sites of MG and TUNEL reaction products are exhibited in orange, a mixture of green and red. Nuclei in the crypt cell show typical “apoptotic bodies” which exhibit intense TUNEL staining. Fig. 5d shows nuclei of midzone cells. Greenish fluorescence is markedly diminished and deviated to the nuclear peripheries which may correspond to heterochromatin. Orange deposits which were based on TUNEL reaction products also localized in the nuclei periphery. Figs. 5b, 6a show the nuclei of tip cells. Orange deposits in the nuclear periphery markedly increased compared with those of midzone cells. A disproportionate decrease of greenish fluorescence was also evident.

The results of CLSM and EM are compared in Fig. 6. In CLSM and EM observations of TUNEL staining deposits in crypt cells (Figs. 6a, b), positive reaction products in crypt cells show fluorescence in the periphery of the nucleus (Fig. 6a, b), which is far more intense than that in other parts of the nucleus (Fig. 6a). That is, only TUNEL-positive reaction products were exhibited at the periphery of the nuclei which corresponds to heterochromatin detected by EM (Fig. 6b).

IV. Discussion

MG is an excellent nuclear counterstain used in light microscopic histochemistry, but can MG-stained substances be detected by CLSM? Actually, the fluorescence generated by MG in CLSM was clearly detectable in the present experiment. Excitation was achieved at 543 nm with a helium neon laser, with an emitted wavelength of 570–590 nm [24]. The membrane fluorescent intensity of MG decreased in the order of dbs-DNA, annealing DNA, nick DNA, and ss-DNA. This indicated that MG predominantly reacts with double-stranded DNA.

Cells in the crypt, which have undergone cell division, move to tip of the villi within 2 days to undergo apoptotic cell death [9]. These results are illustrated in schematic diagrams of the absorptive cell nuclei (Fig. 7). The upper row exhibits localization patterns of MG. The lower row shows localization patterns of TUNEL reaction products. In crypt cells, MG which specifically reacts with double stranded DNA is distributed over almost the entire nucleus. When the cells reach the midzone, MG-stained DNA is shifted toward the peripheral region of the nucleus roughly corresponding to the heterochromatin. The total amount of MG-stained DNA is diminished. In the tip cells, most of the nuclei undergo shrinkage and deformation. In these nuclei, MG-stained DNA is almost totally shifted toward the periphery and the total amount is markedly reduced.

In contrast, these TUNEL reaction products are almost negligible in the nuclei of crypt cells. When the cells reach
Simultaneous Detection of DAB and Methyl Green

In terminal differentiation, the TUNEL reaction products appear and aggregate in the periphery of the shrunken and deformed nuclei of the tip cells. The TUNEL reaction products consistently accumulated in the nuclear periphery, where markedly less MG-stained substances are found. Since the staining labels the nicks formed in the double stranded DNA by endonuclease, it is quite rational to assume that MG-stained DNA diminished at the site intensely stained by the TUNEL method.

The most intense and diffuse MG staining was observed in the crypt regions (base of the villi). While the cells were moving up to the midportions and tips of the villi during the process of differentiation, MG-labeled substances were translocated to the periphery of the nucleus (heterochromatin region) and the MG fluorescence in the heterochromatin region gradually weakened. In contrast, TUNEL reaction products, which are localized to the heterochromatin regions, increased in amount during the process.

The heterochromatin regions are the site of dbs-DNA degradation. The tip showed typical “apoptotic bodies” which exhibited the intense TUNEL staining. In those cells, MG-labeled substances were irregularly fragmented and diminished, and localized along the margins of TUNEL-positive lesions. MG-staining appears to reflect the amount of intact dbs-DNA rather accurately in those damaged cells.

V. Conclusions

1. Methyl green (MG) was found to be valid for nuclear counterstaining even in CLSM.
2. MG preferentially stained intact DNA (undegraded or undamaged).
3. In terminally differentiating cells, “non-transcriptable” DNA appears to shift to heterochromatin regions (periphery of nuclei) to be degraded there (possible apoptotic change).
4. The interrelationship between cell functions, which can be detected by various histochemical observations of cytoplasmic substances, and cellular differentiation state, can be elucidated by using MG in CLSM.

VI. Acknowledgments

This study is dedicated to the late Professor Keiichi Watanabe, one of the co-authors of this study, who often encouraged us to follow the research policy of building a hypothesis first, and then experimenting in order to prove the hypothesis, and using the results as a paper. How sad to think we will no longer be able to benefit from his advice. We offer our prayers for his eternal rest.

This study was presented in part at the 4th Japan-China joint symposium of Histchemistry and Cytochemistry, Chongqing, China in 1996.

VII. References