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Staining of Proliferating Cell Nuclei with Biotinylated dUTP

Tomoyuki Murakami

2nd Department of Pathology, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan
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Abstract A method of in situ biotinylated dUTP incorporation into nuclei was developed for evaluation of cell growth activity.

HeLa cells or frozen sections of cancer tissues were incubated in a solution containing bio-dUTP and the other deoxyribonucleotides (dATP, dCTP, dGTP), after permeabilization of cell membrane with Triton X-100, and signals from bio-dUTP in nuclei were detected with an avidin-biotin-peroxidase complex (ABC method), or with fluorescein-avidin. This staining procedure was simple and rapid as compared with that for BrdU incorporated into DNA.

In HeLa cells, bio-dUTP-incorporated cells could be stained by the ABC method and the labeling index was compatible with that of BrdU, but the intranuclear distribution patterns of bio-dUTP were different from those of BrdU. Flow cytometric detection of bio-dUTP-incorporated cells was also possible. In some frozen sections of cancer tissues, cells synthesizing DNA were labeled with bio-dUTP.

These results suggest that this will be a useful method for evaluating cell growth activity, although the biological significance may be different from the BrdU incorporation experiment.

Key Words : Biotinylated dUTP, BrdU, DNA synthesis, Flow cytometry

Introduction

Evaluation of cell growth activity is important not only for tumor cell biology but also for cancer treatment. The development of a simple and reproducible method for clinical use has long been waited. The conventional methods that utilize a growth curve or ^{3}H-thymidine labeling index are laborious and time-consuming, and the information provided by them have come to be limited for modern biology and medicine. New methods of analysis which have been recently developed include use of flow cytometry (FCM) and immunocytochemistry with monoclonal anti-5-bromodeoxyuridine (anti-BrdU) antibody (1,2), monoclonal anti-PCNA/Cyclin antibody (3,4,5,6) and Ki-67 (7,8,9). In particular, bivariate flow cytometric analysis of BrdU-incorporated cells that allows measurement of DNA synthesis rate vs. DNA content in S phase has brought about a revolutionary progress in the field of cell kinetics (1, 2, 10, 11, 12, 13). However, difficulty is that it requires to label while they are proliferating.

In 1981, Langer et al. demonstrated that

bio-dUTP is effectively incorporated into DNA in vitro (14). Olins et al. showed that isolated macronuclei from Euplotes eurystomus incorporate bio-dUTP specifically into replication band (15). In 1989, Nakayasu et al. studied its incorporation into permeabilized cells and observed that it is incorporated at almost the same sites as BrdU (16). This suggests that the incorporation of bio-dUTP has almost the same biological significance as BrdU-incorporation.

The present investigation was undertaken to compare both methods with aspect to the labeling index and the intranuclear sites of incorporation. In addition, flow cytometric detection of bio-dUTP incorporated cells and staining of frozen tissue sections with bio-dUTP were performed.

Materials and Methods

Cell Culture

Exponentially growing HeLa S3 cells were cultured on chamber/plastic slides (Miles Scientific, Naperville, IL) in Dulbecco's modified Eagle medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% calf serum at 37℃ in a humidified atmosphere of 95% air and 5% CO_2.

Tumor Specimens

Surgical specimens were obtained at Yamaguchi University Hospital. They consisted of a breast cancer, a cancer of the rectum and 2 cases of gastric cancer. A part of each specimen was dipped in OCT compound (Miles Scientific, Napeville, IL) within 3 hours after surgical excision and kept frozen at -80℃ before use. Cryostat sections of 5μm in thickness were made, mounted on slides and put immediately into TBS buffer (10mM Tris-HCl, pH7.4, containing 0.15M NaCl and 5 mM $MgCl_2$).

Other parts of the same specimens were used for BrdU incorporation.

In Situ Incorporation of Bio-dUTP

The method of incorporation was the same as used by Nakayasu et al. (16) except for minor modifications. HeLa S3 cells or tumor sections on slides were washed twice with TBS buffer and then once with glycerol buffer (20mM Tris-HCl buffer, pH 7.4, containing 25% glycerol, 5mM $MgCl_2$, 0.5mM EGTA and 0.5mM PMSF). They were then permeabilized with glycerol buffer containing various concentrations of Triton X-100 (0.5%, 0.05%, 0.01%, 0.005%) for 2 min at room temperature and gently washed three times with glycerol buffer. After that, they were incubated in DNA synthesis buffer (50 mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 0.5mM EGTA, 25% glycerol, 40μM dATP, 40μM dCTP, 40μM dGTP, 30μM biotin-11-dUTP (Enzo Biochem, Inc. N.Y.), 2mM ATP) for 60 min at 37 ℃ and again washed three times gently with glycerol buffer. Cells for negative control were treated in the same way except that they were incubated in the solution without bio-dUTP.

Staining of Bio-dUTP and Flow Cytometry

For microscopic observation, the bio-dUTP-labeled tumor sections and HeLa cells were incubated with avidin-biotin-peroxidase complex (1 : 100 in PBS ; Vector Lab, Burlingame, USA) for 30 min at room temperature. After washing with PBS, the peroxidase was developed with a diaminobenzidine-hydrogen peroxide (DAB, Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution.

On the other hand, HeLa cells for flow cytometry were treated in a similar way but incubated with FITC-conjugated avidin (20μg/ml in PBS ; Vector Lab, Burlingame, USA) instead of avidin-biotin-peroxidase complex. The cells were detached from slide glass and dispersed by gentle pipetting in PBS. Fluorescence intensity of FITC was measured by FACS analyzer (Becton-Dickinson, Sunnyvale, USA). FITC was exited by blue light (485nm) from an arc lamp. Green fluorescence from FITC was collected through a 520 nm long-pass filter and a 530 nm band-pass filter and recorded as the amount of incorporated bio-dUTP. About 5000 cells were measured and a 256-channel histogram was generated.

In Situ Incorporation of BrdU

HeLa cells growing on a slide were labeled with 10μM BrdU (Sigma, ST. Louis, USA) in the Dulbecco's modified Eagle medium. After washed with PBS, cells were fixed with cold 100% ethanol overnight.

Fresh human tumor tissue fragments were labeled in vitro with BrdU according to Sasaki's method (17, 18). Briefly, the tissue was cut into small fragments (1mm^3) with scalpels. The fragments were incubated in Dulbecco's modified Eagle medium containing 20% calf serum and 10μM BrdU for 1 hour at 37℃ under 3 atmospheric pressure of carbogen (95% of O_2 and 5% of CO_2). Then they were washed with PBS and fixed in 100% ethanol for several days. Paraffin

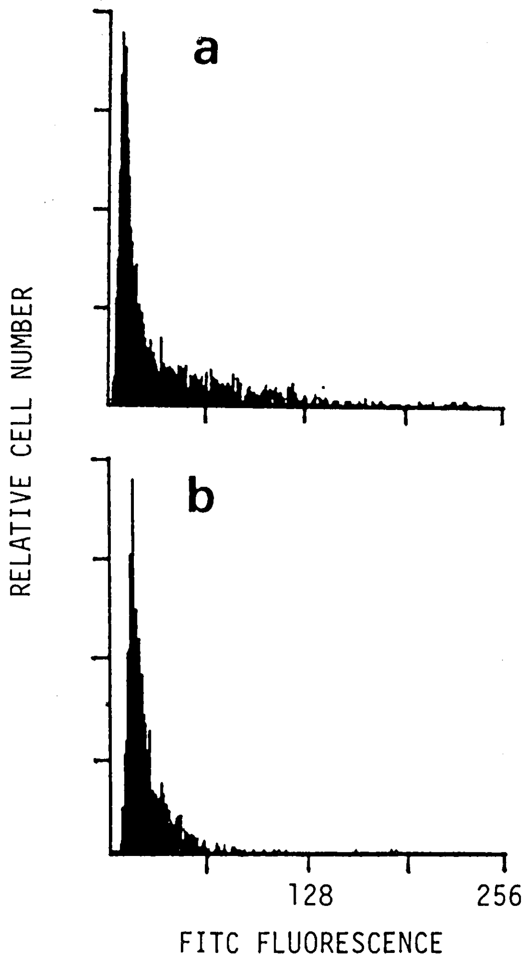

Fig. 3 Fluorescence histograms of HeLa cells labeled with bio-dUTP (a) and of negative control (b). Bio-dUTP-incorporated cells whose fluorescence intensity is higher than that of negative control are identified.

sections of them were made.

Staining of BrdU

The cells or the tissue sections which were labeled with BrdU and mounted on slides were dipped in 4N HCl for 20 min at room temperature. After washed with PBS, they were stained immunocytochemically with a monoclonal anti-BrdU antibody (Becton Dickinson, Sunyvale, CA) by an avidin-biotin complex (ABC) method. Briefly, the cells were incubated with the monoclonal antibody (1:100 diluted with PBS containing 0.5% bovine serum albumin [Dako, Glostrup, Denmark]) for an hour at room temperature and washed with PBS. Then the cells were treated with biotin-labeled second antibody and the avidin-biotin peroxidase complex (Vector Lab, Burlingame, USA) according to a conventional protocol and then developed with DAB solution.

Results

Bio-dUTP incorporated HeLa cells could be easily identified only after treatment with 0.05% Triton X-100 (Fig. 1a). With other concentrations of Triton X-100, positive cells could hardly be detected microscopically because of weak stainability.

The labeling indices of HeLa cells obtained by using bio-dUTP were a little lower than that of BrdU (Fig. 1a, 1b).

The intranuclear distribution of bio-dUTP could be classified into three patterns; nucleolar pattern, diffuse fine-granular pattern and mixed pattern (Fig 2a, 2b, 2c). The distribution pattern of incorporated BrdU were different and could be classified into three patterns; nucleoli-unstained pattern, diffuse fine-granular pattern and diffuse coarse-granular pattern(Fig 2d, 2e, 2f).

As compared with the result of BrdU-labeling, FITC of bio-dUTP-labeled cells was much weaker under the fluorescence micro -scopy (data not shown). However, positive cells could be distinguished by flow cytometry (Fig. 3). This flow cytometric method gave slightly lower labeling indices than those obtained by the ABC method.

Frozen sections of a breast cancer and a gastric cancer labeled by this method was stained successfully (Fig 4). In those preparations some tumor nuclei were stained but stromal cell nuclei were not. However, non of the cells of a gastric cancer and a cancer of rectum were stained despite that BrdU-labeling indices were 10.7% and 22.8%, respectively.

Discussion

The results presented here suggest that

bio-dUTP incorporation in situ can be used as a means of evaluating DNA synthetic activity of cells. However, this method is different from BrdU method in that the latter requires a full set of enzymes and nutrients for cell proliferation as well as oxygen, whereas bio-dUTP is incorporated if DNA polymerases are active and dATP, dCTP and dGTP (dNTPs) are given (14). This may be the reason why frozen sections were labeled with bio-dUTP.

In the absence of ribonucleotide triphosphates (rNTPs), DNA sythesis at initiation points does not start and Okazaki fragments are not generated, because they require RNA primers (19, 20). However, elongation of DNA strands is continued if dNTPs are given. Therefore, bio-dUTP molecules are considered to be incorporated preferentially into leading strand. On the other hand, BrdU molecules are incorporated into leading strands and lagging strands (Okazaki fragments) equally. The different stainability of bio-dUTP and BrdU in the present study may be partly due to the different manner of their incorporation into DNA. Another possibility is that the rate of polymerization is lowered in permeabilized cells due to inactivation of enzymes or other protein factors. Van der Verden et al. thought that DNA sythesis rate declines in such cells, not by the failure to generate Okazaki fragments, but by the decline of the polymerization reaction (21).

The experimental procedure for cell permeabilization should be most carefully controlled. Bio-dUTP molecules do not pass through the intact membrane. If, however, the membrane is highly permeabilized, some enzymes and factors for DNA synthesis will be lost from the cell. It was found in the present investigation that a suitable concentration of Triton X-100 for permeabilization and a suitable time of incubation were 0.05% and 2 minutes, respectively. At higher or lower concentrations, cells were not stained. The fact that concentration of detergent is important was also recognized by Velden et al. (21) and Seki et al. (22). However, the concentration we used was not the same as that used by of Seki or Nakayasu (16). The optimal concentration may depend on cell lines.

The bio-dUTP method seems to be superior to BrdU method in three respects. First, the use of frozen sections allows to study prognosis retrospectively. Second, when applied to cells in suspension for flow cytometry, much less cells are lost by this method as compared to the BrdU method. By the latter method, a large number of cells are lost during treatment with HCl and repeated centrifugations. Third, the process is simple and rapid, because neither cell fixation nor DNA denaturation is needed.

Nevertheless, there remain several points to be improved for clinical use. The one is to make this method more sensitive and it will be achieved by a combined use of FITC-avidin and biotinylated anti-avidin antibody (23). Another is to increase reproducibility of staining. In 2 of 4 cases of cancers, none of the cells incorporated bio-dUTP, although some cells incorporated BrdU. The cause of this discrepancy is not clear, but it seems possible that the time from resection to freezing, the way in which the specimens are frozen and thawed and the concentration of Triton X-100 have a strong influence on the stainability.

This method will be a simple and valuable test for evaluating cell proliferative activity and will give some new information relevant to the mode of DNA synthesis which is not obtained by BrdU method.

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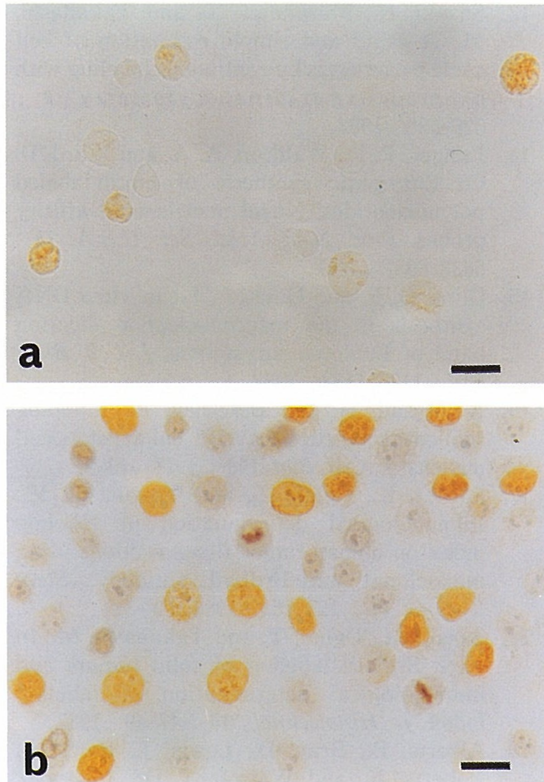

Fig. 1 (a) Bio-dUTP-incorporated HeLa cells as identified by ABC method described in text. The labeling index is 43.8%. (b) BrdU-incorporated HeLa cells as identified by immunocytochemical procedure described in text. The labeling index is 51.0%. Bars = 20μm.

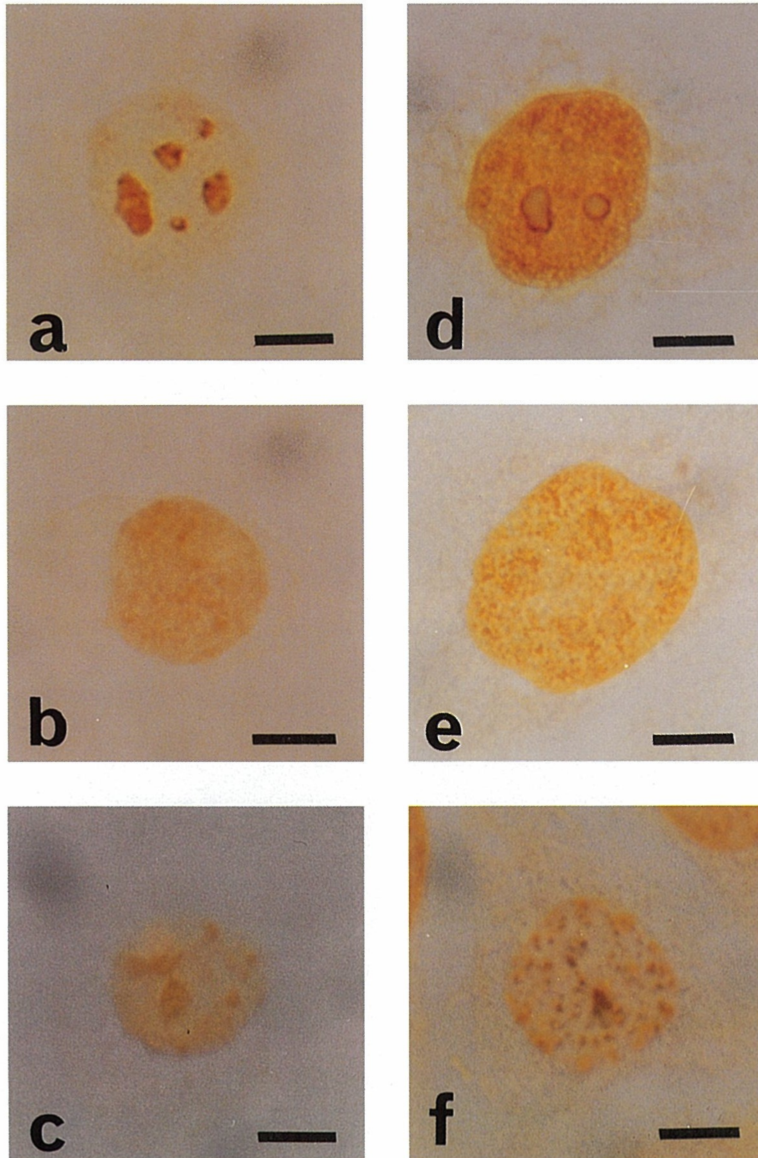

Fig. 2 Distribution patterns of bio-dUTP (a, b, c) and BrdU (d, e, f) in nuclei of HeLa cells. Bio -dUTP distribution patterns: (a) nucleolar pattern, (b) diffuse fine-granular pattern and (c) mixed pattern. BrdU distribution patterns: (d) nucleoli-unstained pattern, (e) diffuse fine-granular pattern and (f) diffuse coarse-granular pattern. Bar = 10 μm

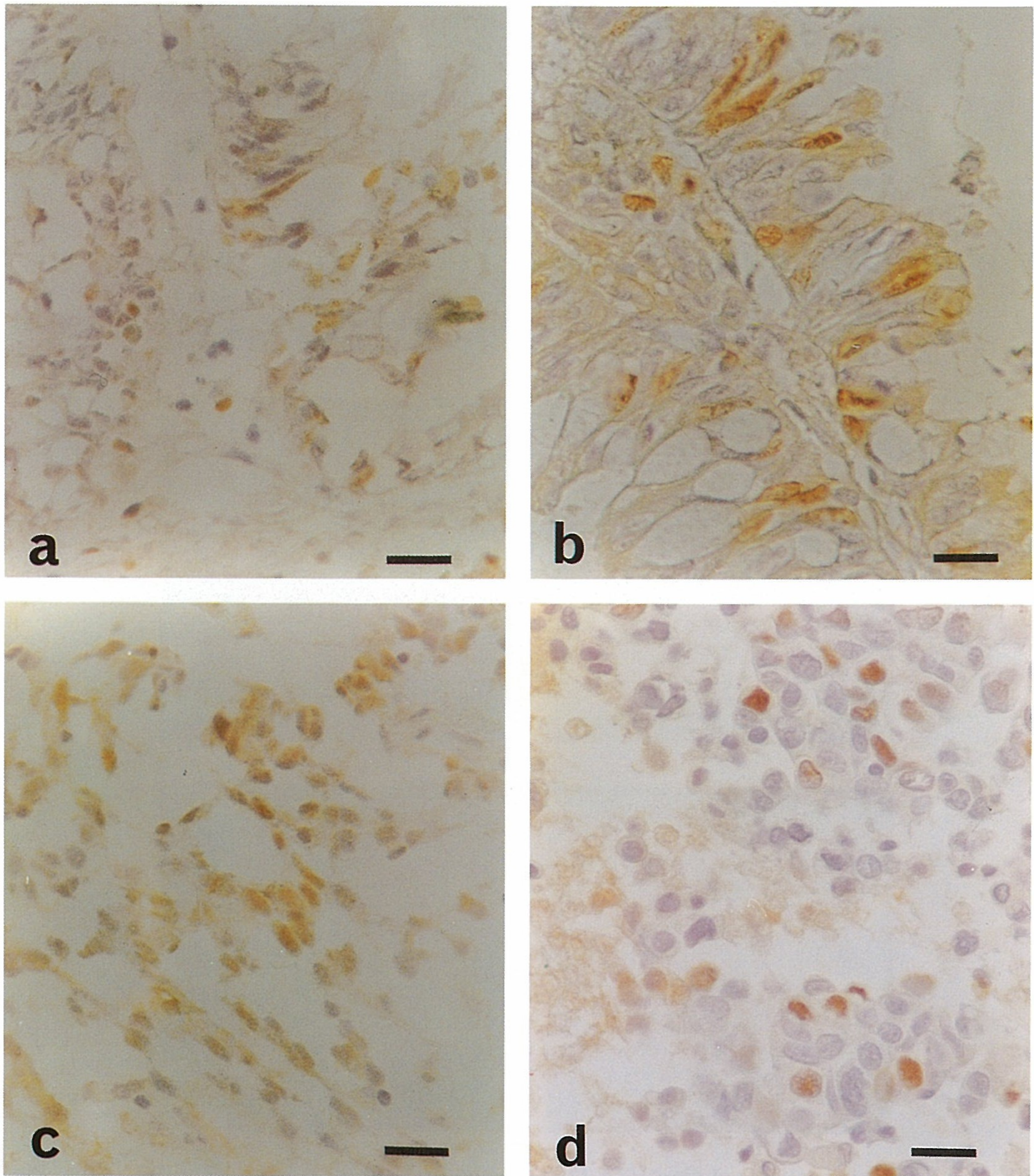

Fig. 4 Sections of gastric cancer (a, b) and breast cancer (c, d). Bio-dUTP-incorporated cells are identified in cryostat sections of gastric cancer (a) and breast cancer (c). BrdU-incorporated cells are identified in paraffin embedded sections of gastric cancer (b) and breast cancer (d). Bars = 20μm