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Improvements in visualisation and localisation of human papillomavirus DNA in CaSki cells by fluorescence in situ hybridization, laser scanning confocal microscopy and three-dimensional image reconstruction

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Abstract

The visual interpretation and localisation of specific DNA sequences in three dimensions in cell nuclei was investigated by fluorescence in situ hybridization (FISH) and laser scanning confocal microscopy (LSCM) using CaSki cells containing 600 copies per cell of human papillomavirus (HPV) DNA type 16 integrated in cellular DNA. Biotinylated DNA probes were used and DNA-DNA hybrids were revealed by a three-step reaction involving a rabbit anti-biotin antibody, a biotinylated goat anti-rabbit antibody and a streptavidin-fluorescein isothiocyanate complex. The DNA from cell nuclei was counterstained with propidium iodide. With standard fluorescence microscopy, some dense fluorescent spots were seen in the cell nuclei. Similarly, with LSCM, some hybridization spots were observed in the cell nuclei but they were at different levels of the nuclei as shown by successive nuclear sections taken along the z axis. The visualisation of multiple hybridization spots confirmed the presence of multiple integration sites of HPV 16 DNA in CaSki cells. Association of LSCM with three-dimensional reconstructions lead to spatial images of hybridization spots obtained by stacking

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(x,y) images from consecutive confocal planes. Rotation of the reconstructed cell nuclei around the y axis makes it possible to distinguish closely adjacent spots. The combination of these techniques improves the detection of hybridization spots and may be of interest to further determine whether the HPV DNA is episomal or integrated in infected cells.

Keywords: CaSki cells; Human papillomavirus; Fluorescence in situ hybridization; Laser scanning confocal microscopy; Three-dimensional imaging

1. Introduction

Under appropriate conditions DNA and RNA sequences can form duplexes with complementary nucleic acid sequences which can be visualized in single cells by in situ hybridization techniques. When nucleic acid sequences are in low quantities in the cells, radioactive probes are usually required for in situ detection [27]. However, the development of non-radioactive probes is now such that in situ hybridization is very sensitive since the labelling of non-radioactive probes and the detection of hybrids have been improved [9,31,37]. DNA sequences as small as 1 to 5 kb can be identified by non-isotopic in situ hybridization on interphase nuclei or on metaphase chromosomes [4,15]. To detect nucleic acid hybrids the probes used are covalently coupled to haptens such as biotin [13] or digoxigenin [7]; they can also be modified chemically by 2,4-dinitrobenzaldehyde [35], 2-acetylaminofluorene [19], mercuration [15,16] or sulfonation [11]. However, the most commonly used labellings are biotin and digoxigenin. Highly sensitive immunoenzymatic or immunofluorescent methods of amplification are applied to reveal nucleic acid hybrids [13,17,41]. Fluorescence in situ hybridization (FISH) has been especially useful for the localisation of infectious agents [24], chromosome sequences [4] and even single copies of genes [18], with conventional fluorescence microscopy. Under similar conditions, human papillomavirus (HPV) DNA, which is 8 kb, can be routinely identified both on tissue sections and cell smears with non-isotopic methods [36]. In addition, the use of different fluorescent markers permits simultaneous investigations on various nucleic acid sequences [25,32].

Technical developments have resulted in availability of laser scanning confocal microscopy (LSCM) equipment which has improved cell visualisation. LSCM has several advantages over conventional fluorescence microscopy, mainly in terms of its greater resolution, elimination of fluorescence background [42] and possibility of 3-D reconstruction [38,39].

HPVs form a heterogeneous group of viruses with more than 70 different types [10]. Some HPV types, commonly found in the genital tract, such as HPV 16, have a high risk of inducing cancers [43]. In many cases, the viral DNA is episomal and in advanced stages of cervical carcinoma the viral DNA is sometimes integrated into the cellular genome [20]. Furthermore, the quantity of HPV DNA detected in HPV-induced lesions may be related to their aggressivity.

In this paper, we evaluated the advantage of LSCM examination associated with

3-D reconstruction [33,34] as compared with conventional fluorescence microscopy for the detection and localisation of HPV DNA type 16, in CaSki cells derived from a human cervical carcinoma. FISH and biotinylated probes were revealed by streptavidin-fluorescein [13,22], whereas the cell nuclei were simultaneously stained with propidium iodide.

2. Materials and methods

2.1. Cells

CaSki cells, derived from a uterine carcinoma [30], were shown to contain 500–600 copies of HPV 16 per cell [29]. They were grown as monolayers in DMEM medium (Gibco, Gaithersburg, MD, USA) with 10% fetal calf serum and antibiotics (penicillin and streptomycin at 10^2 units/ml). At confluence, after 3–4 days, the cells were trypsinized and passaged. For cell deposits, the pellet was resuspended in phosphate-buffered saline (PBS, Merieux, Lyon, France) after washing in PBS. Two deposits of 40 000 cells were applied per glass slide, air-dried at room temperature, fixed in cold acetone for 10 min and stored at −20°C.

2.2. Fluorescence in situ hybridisation (FISH) and detection of DNA-DNA hybrids

Fluorescence in situ hybridization was performed under stringent conditions (Tm 19°C) as previously described [13] using genomic probes of HPV types 16 and 18, included in the plasmid pBR 322. DNA-DNA hybrids were detected by indirect immunofluorescence with a three-step reaction; the slides were successively incubated with an antibiotin rabbit antiserum (Enzo, New York, NY, USA) diluted to 1:500 in 2 × SSC-0.75% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) for 1 h at 37°C, a biotinylated goat antirabbit antiserum (Enzo, New York, NY, USA) diluted to 1:500 in 2 × SSC-0.75% BSA for 1 h at 37°C and a streptavidin-fluorescein complex (Zymed, San Francisco, CA, USA) diluted to 1:30 in PBS. After washing in PBS, the slides were mounted in polyvinyl alcohol containing propidium iodide at 0.5 μg/ml final concentration to counterstain cell nuclei and 2.3% (w/v) 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Sigma, St. Louis, MO, USA) an antifading agent. In control CaSki cells, the HPV DNA 16 or 18 probes were omitted and HPV type 16 probe was replaced by HPV type 18. The slides were stored at +4°C in the dark until examination. Positive reactions were characterized by green fluorescent spots in the cell nuclei.

2.3. Microscopic examination

FISH spots were successively examined under an epifluorescence Zeiss microscope and a Zeiss confocal laser microscope (Model LSM 10), equipped for epifluorescence microscopy. The objective magnification was 63-fold with 1.4 numerical aperture Plan Apochromatic oil immersion objective for high resolution. Fluorescein (λ Ex Max: 494 nm, λ Em Max: 517 nm) and propidium iodide (λ Ex Max: 536 nm, λ Em Max: 623 nm) were excited at 488 nm with an air-cooled argon laser. The laser beam was attenuated with an F1 neutral density filter (10% transmission). The fluorescence signal from both fluorochromes was recorded simultaneously in a single

scan, thus precluding pixel shift between the images. The green fluorescence was collected through a 530/15 nm band pass filter and the red fluorescence through a 590 nm long pass filter. Typically, optical sections were obtained at 0.5-μm intervals along the optical axis. Each plane consisted of 256 × 256 pixels. Photographs were taken with a camera having 35-mm optics.

2.4. Three-dimensional reconstruction

The three-dimensional reconstructions were made using a Compaq DeskPro (386.20 MHz) microcomputer fitted with an MVP-AT imaging board (Matrox, Canada). The software package was written in C language [28]. Prior to reconstruction the 3-D data sets were filtered using a 3-D median filter to remove noise. The surface of the nuclei and of the fluorescent spots were extracted with a 3-D segmentation procedure. The grey level histograms were calculated for each channel. The segmentation thresholds were selected on the basis of the histograms. The surface extraction was obtained with the following neighbourhood rule. For instance, a nuclear voxel (i.e. whose grey level was greater than the threshold) was considered to belong to the nuclear surface if at least one of its 26 direct neighbours (3 × 3 × 3 neighbourhood) did not belong to the nucleus. Different views at different rotation angles around the y axis were generated (22.5°, 45° and 67.5°, respectively). Every surface voxel was projected in a depth buffer (Z-buffer) using a 3-D transformed matrix. The depth buffer was scanned pixel per pixel and the normal vector to each pixel was calculated. The shading of the reconstructed objects was obtained using a simple illumination model for the surface rendering based on the dot products of the normal vectors and the illumination vector [5].

3. Results

3.1. Examination of CaSki cells by standard fluorescence microscopy after FISH

Using an HPV type 16 biotinylated DNA probe, intense fluorescent spots of different sizes were observed at various positions in the nuclei of CaSki cells counterstained by propidium iodide (Fig. 1) and, in some cases, at the nuclear membrane. No fluorescent signal was seen in the cytoplasm. Similarly, in control cells, when the

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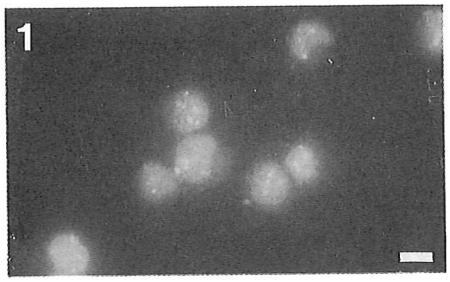
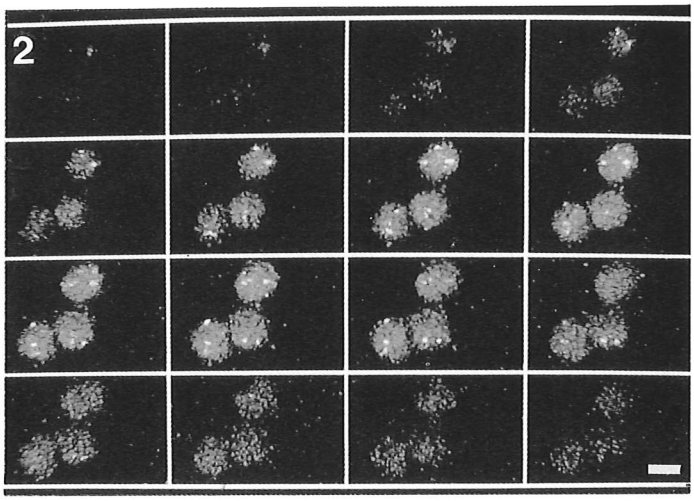
Figs. 1–3. Detection of HPV DNA type 16 in CaSki cells was performed with a biotinylated probe by fluorescence in situ hybridization. The nuclei were counterstained by propidium iodide.

Fig. 1. Standard fluorescence microscopy. Bar = 13 μm.

Fig. 2. Laser scanning confocal microscopy. Sixteen optical sections of nuclei were taken at 0.5-μm intervals along the z axis. Bar = 13 μm.

Fig. 3. Three-dimensional image reconstruction. Three-dimensional images were obtained by stacking up the consecutive sections obtained in Fig. 2 and were observed under different angles by rotation. A: image in the y-z plane; B, C and D: images corresponding to rotations of 22.5°, 45° and 67.5°, respectively, around the y axis. Three-dimensional localisation of the spots is improved if each pair of images A-B and C-D is observed with a reflecting stereoscope. The small arrows point to hybridization spots of irregular sizes and shapes in A and successive rotations in B, C and D. Bar = 13 μm; large arrows point to large hybridization spots corresponding to multiple dots.


1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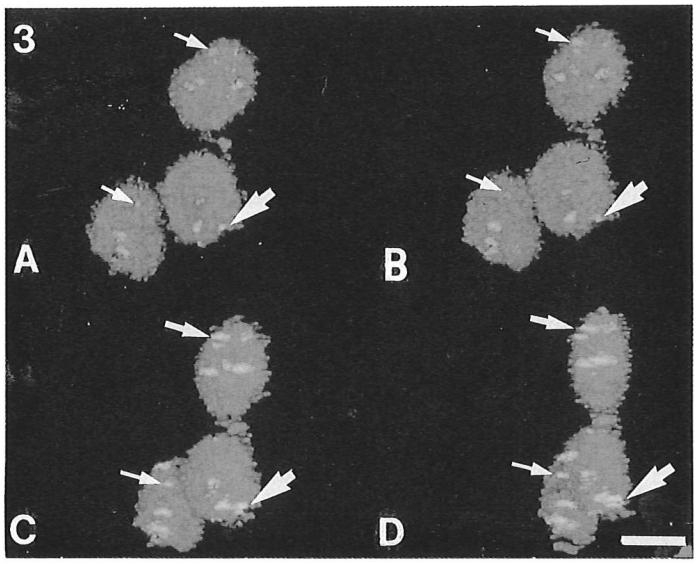
3
A
B
C
D

probe was omitted or when an HPV type 18 DNA probe was used, no fluorescent signal was ever detected. Although the three-step detection method produced very low background staining, it is sometimes difficult to distinguish closely adjacent dots.

3.2. Examination of CaSki cells by laser scanning confocal microscopy after FISH

By examination with LSCM, several hybridization spots with a bright fluorescence were seen in CaSki cells. Optical sections, taken along the z axis at 0.5-μm intervals in CaSki cell nuclei showed distinct compact hybridization spots of various sizes in different nuclear planes (Fig. 2), some of them being in the vicinity of the nuclear membrane.

3.3. Spatial visualisation of HPV DNA type 16 in CaSki cell nuclei by three-dimensional imaging

To improve the visualisation of HPV DNA hybridization spots and to define their spatial position, a three-dimensional reconstruction of CaSki cell nuclei was performed by stacking up the consecutive sections obtained by LSCM in Fig. 2. The reconstructed image was submitted to rotation around the y axis (Fig. 3): image A is represented in the y-z plane, images B, C and D correspond to rotations from 22.5°, 45° and 67.5°, respectively, around the y axis. These images are of interest since hybridization spots of different size produced a clear signal (Fig. 3; small arrows): when adjacent large signal tend to coalesce, they become indistinguishable by standard fluorescence microscopy, but they are seen as double or triple dots by 3-D reconstruction (Fig. 3, large arrows).

4. Discussion

The interest of the present study is to visualize spatially HPV DNA type 16 detected by FISH in CaSki cells using LSCM and three-dimensional image reconstruction as compared with standard fluorescence microscopy. Such an association has already been shown to improve visualisation of subcellular components such as microtubules [42] or microfilaments [40] in various cell types, but it has not yet been used to detect HPV DNA by in situ hybridization. The specificity of FISH in CaSki cells was demonstrated by the absence of fluorescent staining with HPV 18 or any other HPV DNA probe as previously discussed [13]. The sensitivity of HPV DNA detection was improved by combination of FISH and LSCM examination, since one to two copies of HPV DNA 16 were observed in SiHa cells [23] whereas by standard fluorescence microscopy, the detection was limited to 500–600 copies of HPV 16 in CaSki cells [13]. The simultaneous staining of nuclei by propidium iodide and the fluorescein marker of viral DNA hybrids permitted not only the detection of HPV DNA but also the localisation of fluorescent hybridization spots in the nuclei. The main advantage of LSCM is to produce excellent epifluorescent images in which out of focus fluorescence is eliminated. Thus, by LSCM, it could be confirmed that some spots which are in the vicinity of the nuclear membrane by standard fluorescence microscopy, were inside but at the periphery of the nucleus. The presence of multiple

fluorescent spots probably reflects the multiple integration sites of HPV DNA reported in the genome of CaSki cells [6]. Whether LSCM was associated or not to 3-D reconstruction, we have shown that HPV hybridization spots were in different planes of the nuclei. This was so far studied only by immuno-electron microscopy [21] which is time consuming and difficult to perform. In nuclei 3-D reconstruction shows the spatial distribution of hybridization spots with different sizes and shapes. The large dots may represent multiple HPV 16 copies at a single or adjacent chromosomal sites; the size variations may be due to either superimposition of signals from adjacent spots or to the variation in copy numbers per integration site [14]. In the absence of background, as is the case after 3-D reconstruction, the number of hybridization spots detected with 3-D reconstruction may reflect the number of integration sites. Thus, association of LSCM with 3-D reconstruction would be better adapted than conventional methods of microscopy to determine the distribution of hybridization spot number per nucleus of CaSki cells [14]. However, the quantification with LSCM of the number of hybridization spots remains difficult even with adapted stereological methods [8], because of the difficulty in counting accurately small hybridization spots which could just as well be artefacts, especially in cells with small quantities of HPV DNA. An alternative method would be to evaluate the total fluorescence intensity of hybridization spots per nucleus with a highly sensitive video camera, since detector systems open up the way for a quantitative approach using digital imaging microscopy [1,3,26].

In conclusion, this study shows that LSCM associated with 3-D reconstruction appears to be a more powerful method than the conventional fluorescence microscopy and transmission electron microscopy to visualize and localize in CaSki cells HPV DNA 16 hybridization spots having various sizes and shapes. By 3-D reconstruction it was possible to distinguish closely adjacent fluorescent dots. This technique is potentially interesting and more accurate for the examination of infected cells with episomal or integrated HPV DNA. Moreover, the viral DNA being integrated in CaSki cell DNA makes these cells appropriate models to study replication sites of HPV DNA in the different phases of the cell cycle [2,12,38] which are now under investigation.

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