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Autofluorescence spectroscopy of malpighian epithelial cells, as a new tool for analysis of cervical cancer precursors

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Summary. A spectroscopic analysis of autofluorescence was investigated within the cell cytoplasm from cervical malpighian epithelia prepared on Thin-Prep smears. Autofluorescence emission spectra from 22 cervix were analyzed by microspectrofluorometry under a 363 nm laser excitation. Among the analyzed cervix, 6 were in normal limits, 6 in inflammatory limits, 5 were evocative of Low-Grade Squamous Intraepithelial Lesions (LGSILs) and 5 were evocative of High-Grade Squamous Intraepithelial Lesions (HGSILs).

Cytoplasmic emission intensities at 450 nm of cells from inflammatory, LGSIL and HGSIL cervix were equivalent and were 3-fold higher than from normal cervix. All smears presented a two-fold lower autofluorescence emission in the cytoplasm than in the nucleus. The spectral profile analysis allows the discrimination of cells from inflammatory, LGSIL and HGSIL cervix. The 525/425 nm emission ratios were 0.75±0.1, 0.96±0.04 and 1.2±0.1 for inflammatory, LGSIL and HGSIL, respectively. We suggest that smears of normal, inflammatory, LGSIL and HGSIL cervix could be discriminated by the analysis of the 450 nm emission intensity and 525/425 nm emission ratios from cells of malpighian epithelia.

Key words: Autofluorescence, Thin-Prep®, Cervical cancer, Microspectrofluorometry, Malphighian epithelia

Introduction

Cervical cancer is one of the three most common malignant tumors in women in terms of incidence and mortality (Weintraub and Morabia, 2000). The reliability and efficacy of a cervical screening method is measured by its ability to diagnose pre-cancer abnormalities (Corkill et al., 1998). During several years, the Papanicolaou staining was proposed as the most significantly effective test to reduce mortality from cervical cancer. This routine examination for cervico-vaginal diagnosis detects endocervical cells among other epithelial cells (superficial and intermediate malpighian cells) and identifies them either as "cancer" or as "pre-cancer" cells. In addition, the interpretation could be affected by the presence of blood, mucus, obscuring inflammation and air-drying artifact (Aponte et al., 1995; Lee et al., 1997 ; Corkill et al., 1998).

Thin-Prep (Cytyc Corp., Boxborough, Massachusetts, USA), a thin monolayer cytological slide test resulted in a significant increase for the detection of cervical cancer precursors (Papillo et al., 1998; Diaz-Rosario and Kobawat, 1999; Wang et al., 1999). This method of sample preparation has led to an improved diagnosis of low- and high-grade squamous intraepithelial lesions (Bernstein et al., 2001), and to a reduced frequency of false negative results (Papillo et al., 1998; Ashfaq et al., 1999). In addition, results of the Thin-Prep cytology analysis have been recently correlated with the detection of cancer-associated types of Papilloma Virus (HPV) (Sherman et al., 1998 ; Hutchinson et al., 1999).

Fluorescence spectroscopy of endogenous tissue autofluorescence offers an effective, non-invasive and non-destructive approach to the detection of cancer in multiple organ sites (Ingrams et al., 1997). The autofluorescence emission of living cells or tissues corresponds to the emission superimposition from different fluorophores (collagen, tryptophan, reduced nicotinamide adenine dinucleotide and flavin molecules), according to the excitation wavelength (Anidjar et al., 1996; Koenig et al., 1996; Andersson et al., 1998; Dellinger et al., 1998; Bondza et al., 2001). Previously, the analysis of autofluorescence has been applied to the *in situ* diagnosis of different tissue cancers (Anidjar et al., 1996; Koenig et al., 1998). For instance, this methodology has the potential to improve the detection of *in situ* intraepithelial neoplasia and cervical pre-cancer lesions. In cervical biopsies, the

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autofluorescence from human poly- and mononuclear leukocytes has been characterized (Brookner et al., 2000; Heintzelman et al., 2000). Diagnostic algorithms have been developed to classify tissues as normal or abnormal based on fluorescence emission spectra measured *in vivo* (Lohmann et al., 1989; Ramanujam et al., 1996). These results were consistent with histopathological changes associated with cervical dysplasia from biopsies. Moreover, spectroscopic methods show a potential for differentiating cervical neoplasia from the normal uterine cervix (Lohmann et al., 1989; Ramanujam et al., 1994). In the present study, our purpose was to analyze autofluorescence emission spectra from cells of malpighian epithelia in order to characterize different cervix tissue types, previously classified as normal, inflammatory or evocative of dysplasia.

Material and methods

Cervical smears

Twenty-two women from 20 to 68 years of age undergoing routine screening were included in this study (Table 1). Thin-Prep samples were obtained using a cervex brush® from a gynecological screening. Then, cervical samples were gently rinsed immediately into a vial containing 20 ml of a buffered alcohol preservative solution (Preservcyt). All the samples were sent to the Histology and Cytology Laboratory Pol Bouin Institute (Reims CHU, France) for Thin-Prep preparations.

The Thin-Prep technique is based on the immediate fixation of the entire cytological specimen. The T2000 automated processor (Cytyc Corp., Boxborough, MA) is employed to disperse and homogenize the sample. Then, the processor automatically and uniformly transfers cells in the form of a thin layer to specially designed microscope slides (20 mm in diameter area). For each sample, an unstained smear was prepared for the autofluorescence analysis.

Table 1. Analyzed smears.

BETHESDA CLASSIFICATION	BIRTH DATE	CHARACTERISTICS
Normal	1969	
Normal	1980	
Normal	1982	
Normal	1980	
Normal	1978	
Normal	1982	
Inflammatory	1965	
Inflammatory	1975	
Inflammatory	1969	
Inflammatory	1955	
Inflammatory	1951	
Inflammatory	1964	
LGSIL	1958	Viral lesion aspect; HPV+; condylomata
LGSIL	1934	Carcinoma cicatrix; doubtful aspect
LGSIL	1969	Condylomata aspect
LGSIL	1959	Condylomata origin
LGSIL	1968	HPV+
HGSIL	1977	Benign leucokeratosis, one year ago OP
HGSIL	1959	No necrosis, inflammatory cervix
HGSIL	1957	Previous CIN type 2; condylomata aspect
HGSIL	1964	Previous CIN type 3
HGSIL	1963	Ectropion; under OP

CIN: cervical intraepithelial neoplasia; HPV+: positive human papilloma virus detection; LGSIL: low-grade squamous intraepithelial lesions; HGSIL: high-grade squamous intraepithelial lesions; OP: oestro-progestative.

Smear classification

Liquid-based cervical smears were examined after a Papanicolaou staining, by an experienced pathologist using conventional cytology. Smears were classified into four groups according to the Bethesda system (Massad et al., 2001): 6 within normal limits (normal), 5 evocative of Low-Grade Squamous Intraepithelial Lesions (LGSIL); 5 evocative of High-Grade Squamous Intraepithelial Lesions (HGSIL); and 6 within inflammation. Characteristics of each smear are reported in Table 1: the woman's age, the smear classification (HGSIL, LGSIL, inflammatory or normal) and data about the aspect and the origin of the lesion (CIN, HPV).

Autofluorescence analysis

Autofluorescence emission spectra were analyzed from a confocal section within cells of malpighian epithelia using laser scanning microspectrofluorometry (Dilor, France). As previously described (Millot et al., 1994, 1997), a conventional optical microscope (Olympus BH2) was equipped with a x100 objective lens corrected for axial chromatic aberrations. This allowed the observation of the sample, the focus of a 363 nm laser beam (5 μW) (Ar^+, Spectra Physics), and the collection of the fluorescence emission in the 370-560 nm range through the same optics. The pinehole size was fixed to a diameter of 600 μm, which confers a resolution of 3 μm (z) and 1x1 μm^2 (x,y). A mapping of 30x30 emission spectra was performed for each cell. The position of the sample was controlled by a motorized sample holder. The fluorescence emission was spectrally dispersed with a diffraction grating and was analyzed with an optical multi-channel CCD detector coupled to an image intensifier. A computer simultaneously performed the control of stage motions and the spectral data storage. Emission ratios (500-550/400-450 nm integrated intensities) were calculated to characterize the profile of autofluorescence emission spectra. As emission ratios verify a Gaussian distribution law, mean emission ratios were compared by a Student-t-test.

Results

Spectral images of cells from malpighian epithelia

Phase contrast images (Fig. 1A,D) of epithelial cells

and microspectrofluorometry analysis (Fig. 1B,C,E,F) under a 363 nm excitation wavelength are displayed in figure 1. Presented epithelial cells were provided from a dysplastic (HGSIL) cervix (Fig. 1A-C) and from a normal (Fig. 1D-F) cervix. The intracellular emission (450 nm) from the HGSIL cervix (Fig. 1C) was higher than for the normal smear (Fig. 1F). For the normal cervix, the cytoplasm appeared clearly homogeneous in intensity of 410±80 a.u. (mean ± s.d.), whereas the cell from the HGSIL cervix presented a heterogeneous cytoplasm of 1050±420 a.u. (mean ± s.d.), including areas with enhanced emissions. For both cells, the autofluorescence emission was two-fold higher in the nuclear compartment than in the cytoplasm. Emission ratios (500-550/400-450 nm integrated intensities) are shown in Figures 1B and 1E to characterize spectral variations. For the cell cytoplasm of the HGSIL cervix, the ratio was 1.05±0.11. (mean ± s.d.), whereas this ratio decreased down to 0.67±0.07 (mean ± s.d.) in the cell cytoplasm of the normal cervix.

Features of cytoplasmic autofluorescence spectra

The autofluorescence of different epithelial cells was analyzed under a 363 nm UV laser excitation in the 370-560 nm spectral range. The acquisition of emission spectra was performed within the cytoplasm of 30 epithelial cells from normal, LGSIL, HGSIL or inflammatory cervix. Mean spectra of epithelial cells from the four smear types (normal, LGSIL or HGSIL and inflammatory) were compared (Fig. 2). The 450 nm emission intensity from the normal smear (219±96; mean ± s.d.) presented a lower intensity than from the LGSIL smear (1590±950; mean ± s.d.), the HGSIL

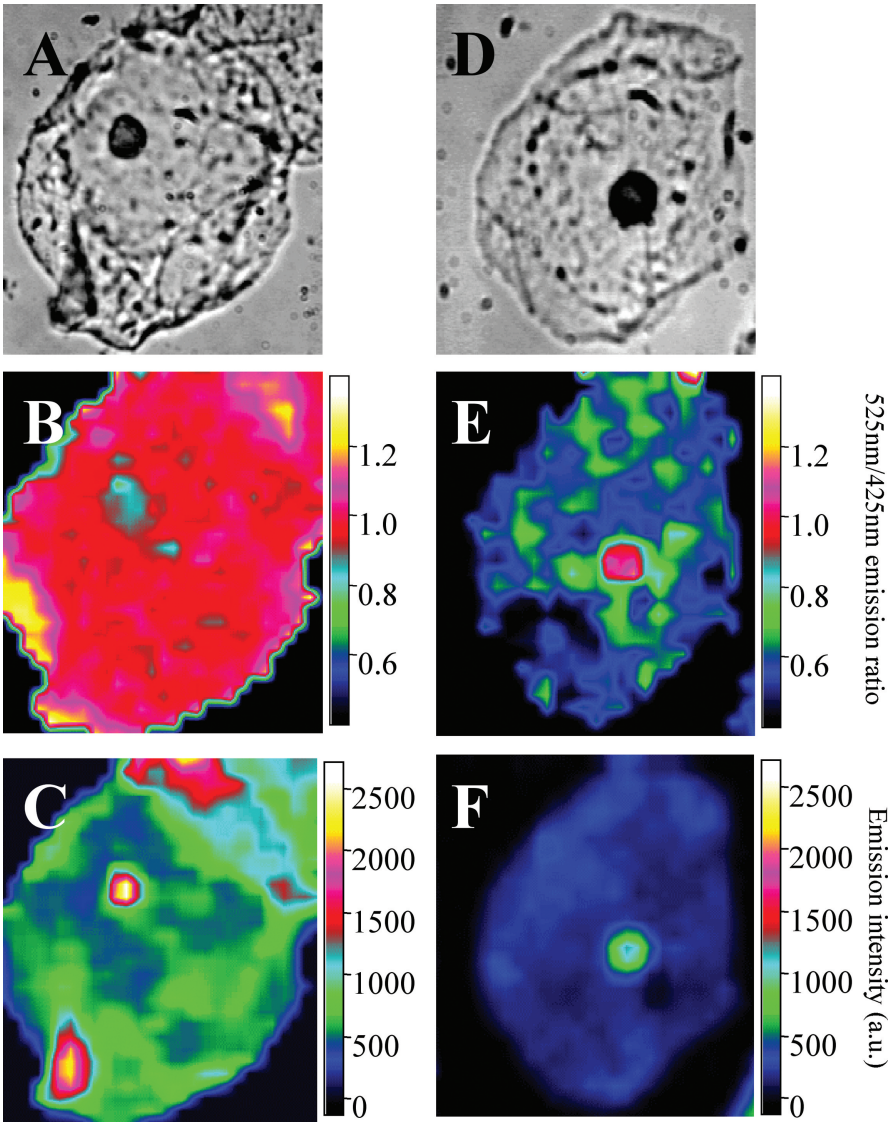

Fig. 1. Autofluorescence images of epithelial malpighian cells from a HGSIL smear **(A, B, C)** and from a normal smear **(D, E, F)**. The presented images correspond to phase contrast images **(A and D)**, to 450 nm autofluorescence emission under a 363 nm excitation **(C and F)** and to emission intensity ratio (500-550/400-450 nm) **(B and E)**.

smear (1650±990; mean ± s.d.) or the inflammatory smear (1110±670; mean ± s.d.). Spectral profiles were characterized by maximum emission wavelengths and 500-550/400-450 nm emission ratios. For cytoplasmic compartments of abnormal smears, maximum emission wavelengths were 442±6 nm, 450±6 nm and 458±5 nm (mean ± s.d.) for analyzed cells from inflammatory, LGSIL and HGSIL smears, respectively.

The variability of cell autofluorescence among malpighian epithelia is presented in Figure 3, according to emission ratios (500-550/400-450 nm) and emission intensities. Cell populations from a normal and a LGSIL smear projected in Figure 3A were well discriminated according to the 450 nm emission intensities. The means ± s.d. were 219±96 and 1590±950 for normal and LGSIL cervix, respectively ($p<0.001$, Mann-Whitney test) (Fig. 3A). On the other hand, cell populations from either an inflammatory or a HGSIL smear were well discriminated according to emission ratios, which were 0.661±0.07 and 1.148±0.08, respectively ($p<0.001$, Student-t test) (Fig. 3B).

Mean autofluorescence spectra from each analyzed smears are presented in the Figure 4, according to the emission ratio and the 450 nm emission intensity. Means of each smear were included in 95% confidence ellipses. Cells from normal smears could be characterized by a low fluorescence intensity (lower than 600 a.u) as compared with cells from abnormal smears. Moreover, mean emission ratios of LGSIL (0.96±0.04) were significantly different from HGSIL (1.2±0.1; $p<0.01$) and inflammatory smears (0.75±0.1) ($p<0.01$). Cells from LGSIL cervix could be characterized by intermediate emission ratios between inflammatory and HGSIL cervix.

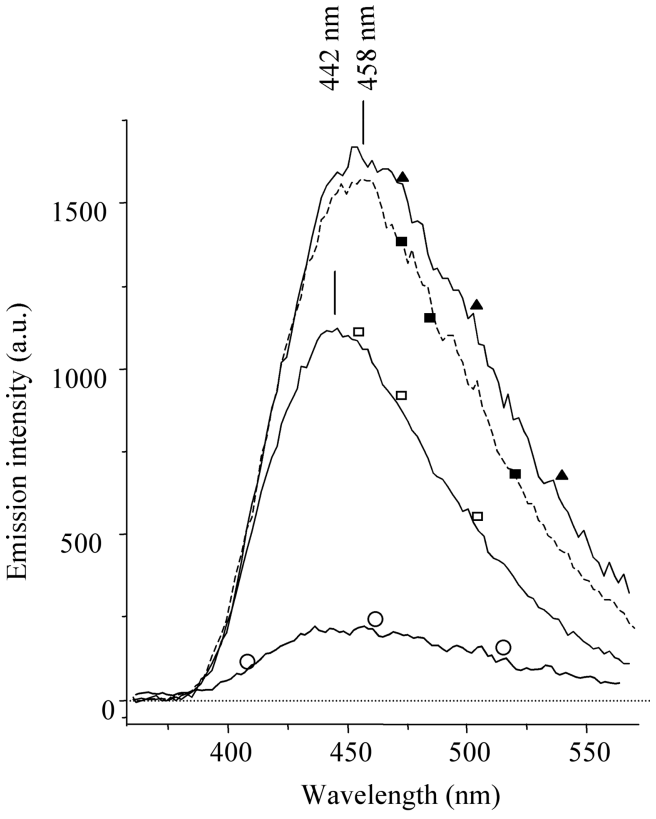

Fig. 2. Autofluorescence spectra of epithelial cells of normal (white circle), inflammatory (white square), LGSIL (black square) and HGSIL (black triangle) smears. Each presented cytoplasmic spectrum is the mean from 30 different cells from the same smear.

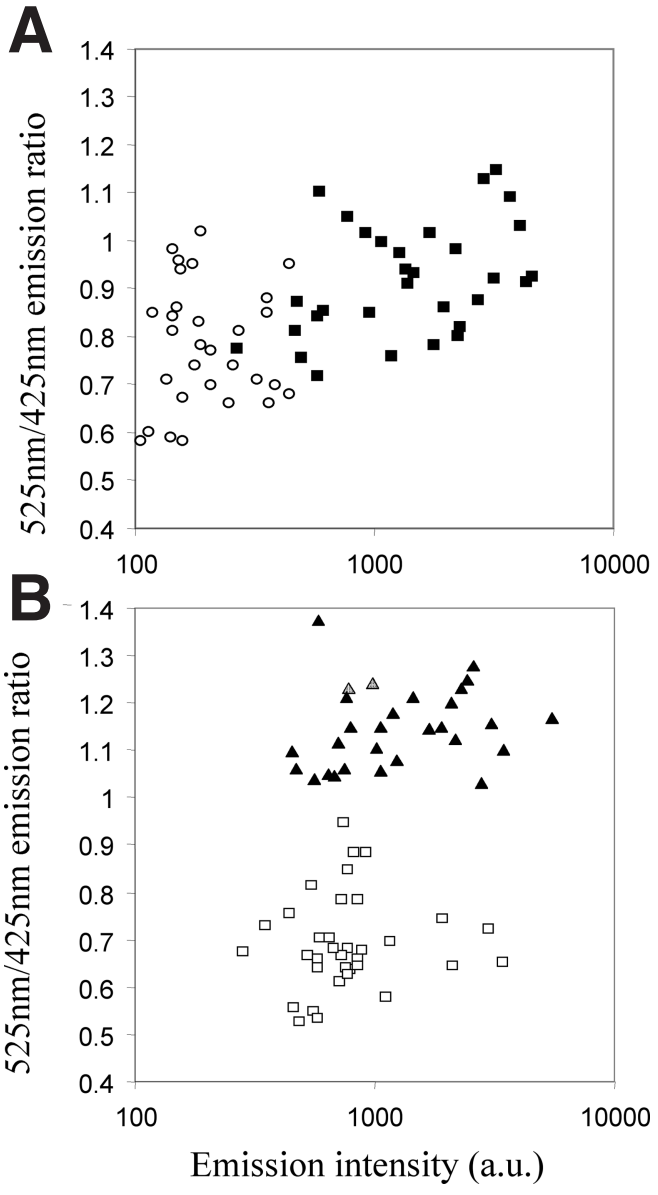

Fig. 3. Emission ratio and 450 nm emission intensity of cytoplasmic autofluorescence from 4 cervical smears. **A.** Normal (white circle) and LGSIL (black square) smears. **B.** Inflammatory (white square) and HGSIL (black triangle) smears. Each point corresponds to the mean cytoplasmic spectrum of one individual cell.

Discussion

Human cancer diagnosis of cervical smears is currently based on the identification of cancer cells. However, the identification of cancer cells by pathologists remains difficult due to the rarefaction of these cells. The presence of inflammation, a common pathology of epithelial tissues, could also result in misclassifications and could reduce the accuracy of analyses. It has been reported that inflammation may be related to the initiation of cancer, due to the damage from the oxidative activity of inflammatory cells (Weitzman and Gordon, 1990).

The analysis of Thin-Prep cytology is considered as superior screening method compared to conventional smears and provided improved sensitivity in the detection of abnormalities (Linder, 1998; Bernstein et al., 2001; Monsonego et al., 2001). Other analyses can be associated with this conventional assay, such as image cytometry which requires a Feulgen staining. The human papillomavirus (HPV) detection can be associated to better discriminate smears with high risk of cervical lesions (Lorenzato et al., 2001). Recently, the ploïdy and the nuclear morphometry of Feulgen-stained cell nuclei of cervical smears have been analysed (Nemec et al., 2002). These results allowed to establish a well discriminative model between HGSIL and normal specimens. The nuclear chromatin pattern from epithelial cells has been described to be highly informative in term of smear classification.

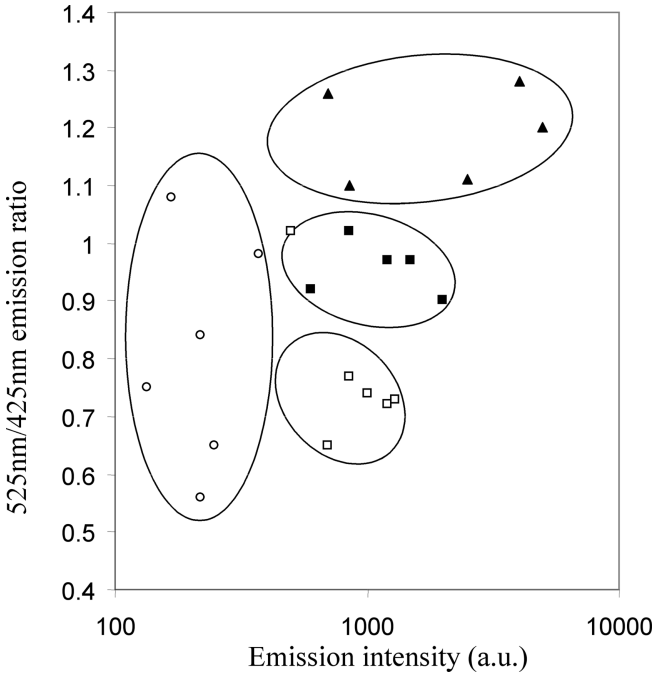

Fig. 4. Means of autofluorescence emission of classified smears as normal (white circle), inflammatory (white square), LGSILs (black square), HGSILs (black triangle). Means from cell cytoplasms (n=30) are presented according to the (500-550/400-450 nm) ratio value and the 450 nm fluorescence emission.

We attempted here to characterize cervical epithelial cells by the autofluorescence analysis of emission spectra, without cell staining. The discrimination between cells from normal and abnormal smears (HGSIL, LGSIL and inflammatory) has been performed according to emission intensity values. HGSIL, LGSIL and inflammatory were discriminated in terms of emission ratio (500-550/400-450 nm). The autofluorescence distribution appeared different according to the smear origin and displayed an additional cytoplasmic component visualized on inflammatory or on dysplastic smears whatever the lesion grade. This component was characterized by a high emission intensity associated with a shifted spectrum about 500 nm. To enhance the precision of the smear classification, the number of analyzed cells would be higher, e.g. 200 cells. Instead of the whole emission spectrum, dual wavelength emission images at 425 nm and 525 nm would allow to increase the analyzed cell number in a given delay and to improve the classification.

Autofluorescence emission spectra depend on the fluorophore concentration and the excitation wavelength (Banerjee et al., 1998). Under 363 nm UV excitation, it has been suggested that most, if not at all, of the cell autofluorescence arises from the contribution of the NAD(P)H,H^+ intracellular coenzymes (Andersson et al., 1998; Dellinger et al., 1998; Bondza et al., 2001). Fluorescence emission from living or dead cells was produced by equivalent fluorophores. However, the results reported here would not be applied directly to an *in vivo* diagnosis from living cells, in reason of differences between living and dead cell fluorescence. Further investigations would be performed to determine spectral features of living epithelial cells from different cervix. Previously, it has been demonstrated that during the development of neoplasia, epithelial tissues undergo biochemical and structural changes (rates of metabolic activity, cell proliferation and/or death) that can correspond to changes in tissue emission (Georgakoudi et al., 2002). Such changes are likely to alter both tissue morphology and biochemistry and could be detected by fluorescence microscopy. Drezek et al. (2001b) have demonstrated that under a 380 nm excitation, cervical tissue fluorescence spectra present characteristic changes associated with dysplasia. Although many studies have reported on different fluorescence emissions between normal and cancer tissues, the origins of these changes have not yet been identified to biochemical changes.

Most autofluorescence studies have also been performed on cervix biopsies. Emission from tissues include data from both cellular and extra-cellular components such as collagen (Heintzlman et al., 2000). NAD(P)H and collagen have been proposed to be quantitative *in vivo* bio-markers of pre-malignant change in uterine cervix dysplasia (Georgakoudi et al., 2002). Drezek et al. (2001b) have demonstrated that the NADH emission, which increases with cervical dysplasia, would

correspond to a diagnostic indicator. Model spectra were consistent with clinical measurements and indicated that differences between normal and dysplastic tissues would be dependent on an increase in NADH emission and a decrease in collagen emission. However, the described model predictions were sensitive to the patient age and the epithelial thickness (Drezek et al., 2001b). Pattern fluorescence differences may be dependent either on the cervix histological integrity, the dysplasic grade or the inflammatory grade of the cervix. Several studies have been performed to elucidate the relationship between fluorescence spectra and the underlying tissue biochemistry and morphology (Ramanujam et al., 1994; Drezek et al., 2001a). The cell autofluorescence intensity could be considered as an intrinsic probe of the cell metabolism (Mayevsky, 1994). Although biochemical changes would precede architectural changes during the progression of the dysplasia sequence or during the onset of inflammatory response (Heintzelman et al., 2000), the relation between the tissue properties and the resulting spectra remains poorly understood.

In conclusion, the study of autofluorescence spectra from epithelial cells could be considered as a new tool for the analysis of cervical smears. These emission spectra would be informative and predictive in terms of smear classification and may complete the actual diagnosis method which is based on dysplastic cell screening.

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