Cell Surface Characteristics and the Detection of (Pre)malignancy of the Human Uterine Cervix

Hans Davina

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Tekening omslag:
Schematische voorstelling van het meerlagig niet-verhoornend plaveiselepiteel van de baarmoederhals. Aangegeven is wat achtereenvolgens is onderzocht: de celoppervlakte-morfologie van vooral de meest oppervlakkig gelegen cellen (hoofdstuk 1 en 2), de celoppervlakte-cytochemie in het dieper gelegen deel van de intermediare zone (hoofdstuk 3) en de lectine-binding van de afgeplatte cellen (hoofdstuk 4, 5 en 6).

Cell Surface Characteristics and the Detection of (Pre)malignancy of the Human Uterine Cervix

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Johannes Herman Maria Davina

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krips repro meppel

Promotores: Prof. Dr. A. M. Stadhouders
Prof. Dr. U. J. G. M. van Haelst
Co-referent: Dr. P. Kenemans

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Aan mijn Ouders
Aan Joop

Contents

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Structural and functional, in particular antigenic cell surface properties have increasingly been the object of cell biological investigation. The cell surface has been shown to play an essential role in processes of regulation of differentiation and histogenesis (Singer, 1974; Bretcher and Raff, 1975, Nicolson et al., 1977), and also in pathological processes as neoplastic transformation and tumorigenesis, tumor invasion and metastasis (Emmelot, 1973; Easty, 1974; Robbins and Nicolson, 1975; Nicolson, 1976; Poste, 1977; Hynes, 1979; Nicolson, 1982; Kenneth et al., 1982).

Cell surface organization and carcinogenesis

The plasma membrane-cell surface complex is defined here as the structure at the external border of the cell, which consists of a lipid bilayer, integral and associated membrane proteins in connection with their oligosaccharide side chains of the glycocalyx, and membrane associated cytoskeletal components (fig. 1) (Alberts et al., 1983). This complex is intimately involved in all interactions between the cell and its environment. It plays a crucial role in cell-cell and cell-substrate interactions during morphogenesis, and in the maintenance of normal cellular functions in differentiated tissue.

During the last decades, evidence has accumulated that abnormal growth and behavior of neoplastic cells is associated with alterations of the plasma

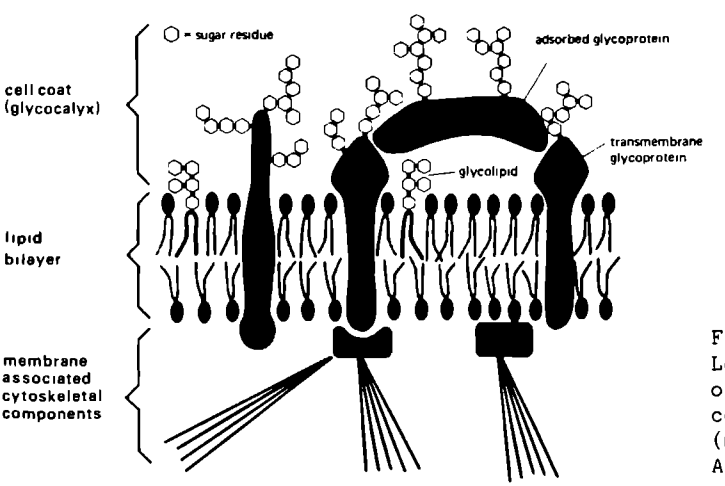

Fig. 1
Levels of organization of the plasma membrane cell surface complex (modified after Alberts et al., 1983).

membrane-cell surface complex (fig. 2) (Lmmelot, 1973; Easty, 1974, Robbins and Nicolson, 1975; Nicolson et al., 1976; Poste, 1977; Hynes, 1979, Kenneth et al., 1982). Early attempts to define the difference between the surface of normal and tumor cells exclusively in terms of a single parameter have failed. It is now apparent that there is a multiplicity of often interrelated changes in the surfaces of cells which have undergone neoplastic transformation (Robbins and Nicolson, 1975). However, certain membrane related aspects of the behavior of malignant cells have emerged which, if not common to all malignant cells, have been detected in many of the systems investigated. In general in malignant cells, there appears to be a deficient contact control of movement and proliferation, as well as deficiencies in contact mediated communication. Reduced cellular adhesion of malignant cells has also long been implicated in tumor invasion and metastasis (Fidler, 1975; Nicolson, 1977; Poste, 1977; Hynes, 1979; Nicolson, 1982). The occurrence of tumor associated receptor sites on the surface of malignant cells may provide a basis for a more specific therapy of cancer. It may then be possible to utilize the aberrant cell surface properties responsible for many of the unfortunate consequences of malignant transformation for its successful treatment (Fasty, 1974).

In summary, the following central theses can be formulated:
(a) the plasma membrane-cell surface complex is of decisive significance for the social behavior of the cell and its alterations like unlimited growth, tumor invasion and metastasis (for reviews see: Singer, 1974; Fidler, 1975;

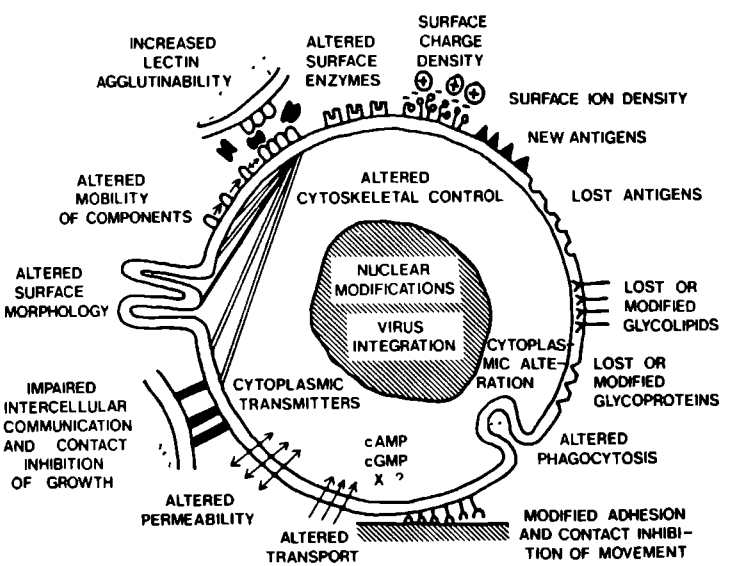

Fig. 2
Schematic representation of some cell surface alterations found after neoplastic transformation (according to Collard, 1976; modified after Robbins and Nicolson, 1975).

Bretcher and Raff, 1975, Nicolson et al., 1976; Poste, 1977, Nicolson, 1977; Hynes, 1979; Nicolson, 1982);

(b) malignant cells are characterized by well defined modifications in cell surface organization (for reviews see: Parsons and Subjeck, 1972, Emmelot, 1973; Wallach, 1975; Robbins and Nicolson, 1975; Poste and Weiss, 1976; Nicolson and Poste, 1976a; Nicolson and Poste , 1976b; Nicolson, 1976; Nicolson et al., 1977; Hynes, 1979, Nicolson, 1982; Kenneth et al., 1982).

In non-clinical cancer research, the altered surface properties are used as criteria for evaluation of the transformation of cultured cell lines by virusses, by chemical carcinogens, or by irradiation. In clinical cancer research however, it is still insufficiently realized that a qualitatively and/or quantitatively altered cell surface organization, expressing altered cell surface properties, offers good possibilities for early diagnosis and treatment. It has been the aim of the studies incorporated in this thesis to analyse the altered cell surface organization of epithelial cells of the uterine cervix related to carcinogenesis using various light and electron microscopical techniques.

Factors of importance in cervical neoplasia

For a number of reasons, the epithelium of the human uterine cervix is an unique object for studies of alterations of the cell surface organization in neoplasia. The uterine cervix offers an appropriate opportunity to study early preneoplastic lesions, because such lesions are accessible to relatively simple sequential visual observations, to atraumatic techniques of exfoliative cytology, and to tissue sampling for histopathological diagnosis by conventional biopsy techniques (Wilbanks, 1976). In addition, there are some other reasons such as the importance for public health, the appropriately developed conventional cytological screening procedures for cervical cancer (Vooijs, 1978) and the local concentration (Nijmegen) of know-how on cervical diagnosis and research.

The uterine cervix is apt to be exposed to a host of agents: infectious (virusses; bacteria), chemical (endogenous and exogenous hormones; sperm DNA, protamines and histones in semen), and physical (mechanical irritation; trauma) ones. Some of these are supposed to be carcinogenic. The cervical epithelium reacts to the injury by undergoing regeneration, hyperplasia, metaplasia, or neoplastic transformation. The transition zone of the cervix,

the site at which the ectocervical squamous and the endocervical columnar epithelium meet, undergoes constant remoddelling from the menarche on. Sexual activity and pregnancy may also be of influence. This area is particularly unstable and susceptible to neoplastic transformation; over 90% of cervical neoplasma arise in this site (Coppleson et al., 1976).

Strong evidence has been presented to link cervical neoplasia to herpes simplex virus type 2 (HSV-2) (Thomas and Rawls, 1978; Aurelian et al., 1981) and human papillomavirus (HPV) (Meisels and Morrin, 1981) infections. The frequently occuring infections with genital HSV-2 and HPV are sexually transmitted diseases. After the initial infection cells in the transition zone of the cervix may retain (part of) the viral genome in a so-called non-productive, latent infection (McDougall et al., 1981). Upon additional pathogenetic stimulation and/or a deficient immune surveillance of the host, these cells may become malignant. In this respect it is of importance that from a relative large number of patients with an apparently normal transition zone, cells can be isolated containing latent form HSV-2 (McDougall et al., 1981). This agent particularly occurs in parabasal and basal cells. Whether or not HSV-2 and HPV act as initiators or promotors of cervical cancer, remains to be settled. Generally speaking at the present time there is no convincing evidence to link such agents as Cytomegalovirus, Trichomonas vaginalis, Chlamydia, nuclear DNA of spermatozoa, smegma, and oral contraceptives to the development of cervical neoplasia (for a recent review see: Fu et al., 1983).

Point of no return in cervical carcinogenesis

A number of intermediate stages between normal epithelium and infiltrative carcinomatous epithelium can be distinguished, i.e. mild, moderate and severe dysplasia, carcinoma in situ and micro-invasive carcinoma (fig. 3). However, the "point of no return" in this sequence can not be pinpointed accurately. Without therapeutic intervention, carcinoma in situ develops into infiltrative cervical carcinoma in more than one third of the cases within a period of 9 years (Peterson, 1956). There is no unanimity about the question whether a carcinoma in situ lesion can regress to dysplasia (Patten, 1978). Prospective cytological studies (Hall and Walton, 1968; Richart and Barron, 1969) have shown that in each of three degrees of dysplasia both regression and progression can occur. The well-known term "cervical intraepithelial neoplasia" (CIN) introduced by Richart (1967), indicates the inability to discriminate between malignant and benign neoplastic cells.

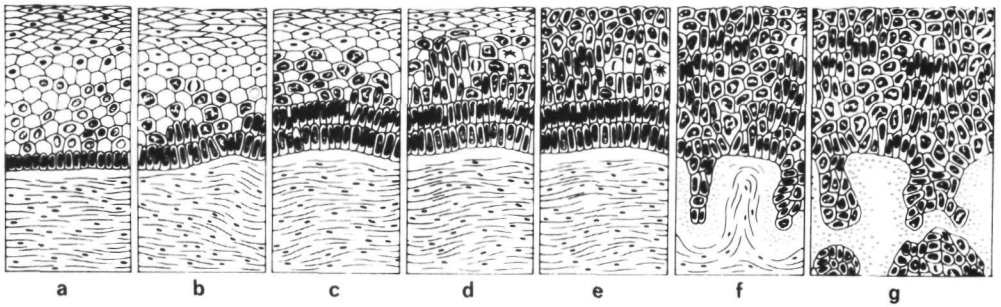

Fig. 3 Schematic representation of precancerous and cancerous lesions of the human uterine cervix. a. normal stratified squamous epithelium, b. mild dysplasia, d. moderate dysplasia, d. severe dysplasia, e. carcinoma in situ, f. micro-invasive carcinoma, g. invasive carcinoma (according to Schuhmann, 1981).

It seems likely that the occurrence of at least part of the atypical epithelial cells in smears is related to inflammation or other non-carcinogenic stimuli rather than to true neoplasia. Epidemiological evidence points out that mild and moderate dysplasias may frequently arise as a non-specific reaction to inflammation and infection (Thomas and Rawls, 1978). The inability to predict the future behavior of a given cervical intraepithelial neoplasia is a consequence of the difficulty that the conventionally used cytological criteria do not allow the discrimination of non-neoplastic from neoplastic changes. However as discussed above it is foreseeable that such discriminating criteria will become available when it is found that apparently normal cells reveal cell surface alterations due to factors which are inducing or promoting for the development of neoplasia. This will then offer important possibilities to the early diagnosis of cervical cancer. Moreover, when cell surface characteristics appear to correlate with clinical stages of aberrant cell behavior (viz. infiltrative growth and metastasis), this will constitute a substantially contribution to the important question of prognosis and pretreatment staging (Nelson and Urcuyo,1976).

Outline of the present investigation

The aim of the studies in this thesis has been the detection of alterations in the plasma membrane-cell surface complex of normal, premalignant, and malignant cervical epithelial cells and to establish their significance for an improved early clinical diagnosis. Morphological surface characteristics of squamous cells have been studied using the scanning electron microscope

(chapters 1 and 2), and cytochemical surface characteristics have been studied by labelling binding sites and using the transmission electron microscope (chapter 3) and the light microscope (chapters 4, 5, and 6).

The development of a pattern of microridges at the cell surface of squamous epithelial cells as observed with the scanning electron microscope (SEM) seems to be indicative of normal cell differentiation (Murphy et al., 1973; Williams et al., 1973; Allen et al., 1976; Rubio and Kranz, 1976). Lack of development of this characteristic pattern could be indicative of an aberrant differentiation or de-differentiation (Williams et al., 1973; Allen et al., 1976; Rubio and Kranz, 1976). Chapter 1 studies the variation in SEM surface structure of the squamous cells in relation to their location within the epithelium. This information is of importance in settling the question of whether or not structural alterations at this level of microscopic resolution can be used as a diagnostic criterion. In chapter 2 attention is focussed on the cancer cells. Based on a review of the relevant literature and on our work an assessment is made of the usefulness of cell surface ultrastructural characteristics as early markers of irreversible neoplastic transformation.

In the plasma membrane-cell surface complex oligosaccharide residues of the glycoconjugates contain specific binding sites for a variety of chemical agents like charged particles, lectins, antibodies and hormones. Tannic acid is a dye which can be used for the ultrastructural visualization of alterations in the carbohydrate-rich glycocalyx of the cell surface. Chapter 3 describes the results of tannic acid binding in the normal and neoplastic squamous epithelium of the uterine cervix.

Lectins are proteins which specifically bind to oligosaccharide residues at the cell surface (Sharon and Lis, 1972). A number of studies has shown alterations in cell surface glycoconjugates during malignant transformation in various tissues (for reviews see: Bhavanandan and Davidson, 1982; Smets, 1982). In chapter 4, a concanavalin A-horseradish peroxidase (Con A-HRP) labelling method for morphologically normal cells in cervical cell suspensions is introduced for the detection of cancer. Instead of a machine-aided assessment of the labelling results, a visual assessment is tested for use as a routine procedure (chapter 5). Finally, the feasibility of the labelling method in the detection of precancerous and cancerous lesions is determined by

labelling cells from patients with mild, moderate and severe dysplasia, carcinoma in situ and squamous cell carcinoma and from treated patients. The Con A-HRP labelling method is then compared with the Papanicolaou method (chapter 6).

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Chapter 1 **Surface Pattern Differentiation of the Epithelial Cells of the Human Uterine Ectocervix**

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Abstract

This review considers the SEM cell surface pattern of the human ectocervical stratified squamous epithelium and its differentiation during migration from the basal to the exfoliating layer The outer surface of flattened superficial cells has been generally characterized by microridges. Although this surface pattern is indeed relatively stable -as follows from an apparently limited sensitivity to preparation factors- it also shows dependency on the hormonal state of the patient. In contrast to the superficial cells very little information is available on the surface structure of the normal intermediate, parabasal and basal cells

The deep surfaces of the superficial cell layers have been examined using a stripping technique, whereas the surfaces of the deeper cell layers have been investigated with a freeze-fracturing technique During migration from the basal cell layer upwards, the following structural parameters change gradually the shape of the cells, the size of the intercellular space and the length and width of the cellular processes As a result of the differentiation process the flattened cells of the superficial zone acquire microridges and crests on the luminal side These interdigitate respectively, with globular microvilli and furrows present on the basal side of the overlying cell Crests and furrows delineate regions on the cell surface in which variable microridge- and microvillus-patterns appear Because crests and furrows are not only present on the cell margins but also more centrally on the surface, the cell surface shows "regional' differences

The functional significance of the cell surface structure is discussed. Whereas the surface processes of the basal and parabasal cells presumably are engaged in the enhancement of intercellular contact and must permit metabolic interchange with the intercellular space, the interdigitating processes of superficial cells probably deal with an increase of the surface adhesiveness and in particular the resistance to sideways movements

KEYWORDS cervix uteri, differentiation, epithelium, exfoliated cells, freeze-fracturing, hormonal state, microridges, microvilli, preparation, stripping.

Introduction

The normal uterine cervix possesses two types of epithelium A one layer thick, cylindrical epithelium lines the endocervical canal, whereas a non-keratinizing stratified squamous epithelium in continuity with the vaginal epithelium, covers the ectocervix A metaplastic change of cylindrical epithelium into stratified squamous epithelium preferentially occurs at the squamo-columnar junction between both types of epithelia. The squamo-columnar junction is also the site where preneoplastic and neoplastic transformation generally occurs. These are indications that the process of epithelial differentiation in this region of the cervix is sensitive to disturbing influences. Conditions that are of likely importance in metaplastic cell differentiation are the hormonal state of the patient and/or the pH of the cervical mucus (Kroeks and Kremer, 1977, Singer, 1977) and various pathological processes such as inflammation.

Several studies have been performed to find out whether or not (pre)neoplastic transformation of the ectocervical cells in biopsies or in cytological smears results in a modified cell surface structure as observable with a scanning electron microscope (SEM) (Jordan and Williams, 1971, Williams et al , 1973a,b, Murphy et al., 1973, Ferenczy and Richart, 1973, Murphy et al., 1974, Ferenczy and Richart, 1974, Bonilla-Musoles et al , 1974, Murphy et al , 1975, Allen et al., 1976, Ludwig and Metzger, 1976, Rubio and Kranz, 1976, Rubio and Finhorn, 1977, Sherman, 1977, Ferenczy and Gelfand, 1979).

This paper considers variation in SEM surface structure of the ectocervical cell in relation to its location within the epithelium, its actual contact with neighbouring cells and its dependency on the hormonal state of the patient The influence of preparation procedures is also discussed. This information is of importance in settling the question of whether or not SEM structural alterations can be used as a diagnostic criterium. Moreover, this information is relevant when applying more advanced methods of examination of the cell surface such as the demonstration of cell surface receptors with labeling techniques

Firstly, a survey of the SEM surface structure of exfoliated and exfoliating normal ectocervical cells will be given. The relation of this pattern with the hormonal state of the patient

SCANNING ELECTRON MICROSCOPY/1981/III (Pages 37-48). SEM Inc., AMF O'Hare (Chicago), IL 60666, USA

will be described. The effects of preparation procedures will be reviewed. Subsequently, two techniques which we used to analyse the cell surface structure in successive cell layers of the ectocervical epithelium, will be described. Lastly, the results obtained with both techniques are reported and discussed.

Surface pattern of exfoliated and exfoliating normal ectocervical cells

Exfoliated cells

Various authors give almost identical descriptions of the surface pattern of exfoliated superficial cells from the non-pathological ectocervix (Murphy et al., 1973, Llanes et al., 1973, Murphy et al., 1974, 1975, Leif et al., 1975, Kjaergaard and Poulsen, 1977). The flat, polygonal cells are large with a diameter of 30-40 µm and they possess a small centrally located nucleus. In the SEM their surface appears highly rugated. Shallow, branching and interlacing convolutions are present, forming a system of microridges which cover the entire cell surface (fig. 1). These microridges are 1-4 µm long, about 0.15 µm wide and are separated from each other by a distance of about 0.25 µm. As a rule no particular orientation of the microridges is observable in the central part of the cell. At the periphery some orientation parallel to the cell boundary is occasionally seen. Little information is available about the SEM surface structure of exfoliated cells from deeper layers than the superficial zone. Only Llanes et al. (1973) have given some data. According to these authors, the exfoliated intermediate cells do not possess a rugated surface, but the cells show stubby, club-shaped, irregularly orientated microvilli. Furthermore they also state that exfoliated parabasal and basal cells are covered with numerous well developed microvilli, which are finer and longer than are those present on the intermediate cells.

Exfoliating cells

The SEM surface structure of the exfoliating cells of the ectocervical epithelium has been studied extensively (Jordan and Williams, 1971, Williams et al., 1973a,b, Nasr et al., 1973, Ferenczy and Richart, 1973, Bonilla-Musoles et al., 1974, Ferenczy and Richart, 1974, Ludwig and Metzger, 1976, Rubio and Kranz, 1976, Rubio and Hinhorn, 1977, Sherman, 1977, Ferenczy and Gelfand, 1979). As is illustrated in figure 2, SEM allows fully differentiated cells to be seen on the top of the ectocervical epithelium. The large, flat, polygonal cells are arranged in an irregular pavement-like pattern. The partially overlying cells appear to be loosely attached to one another, demonstrating various stages of exfoliation. At higher magnification it appears that the surface of exfoliating cells possesses the same microridge pattern as is present on the surface of superficial cells in smears. In addition to the microridge pattern, the cell surfaces show well-defined structural elevations separating cell surface areas. These elevations have frequently been interpreted as cell borders (Jordan and Williams, 1971, Williams et al., 1973a,b, Borilla-Musoles et al., 1974, Fink and Thomas, 1975, Rubio and Kranz, 1976, Sherman, 1977, Lamb et al., 1978, Steger and Hafez, 1978). Ludwig and Metzger (1976) have distinguished not only structural elevations, called crests, but also structural grooves, called furrows. These authors conclude that "microridges and the pattern of cellular border formations ... represent cellular (i.e. microridges) and intercellular (crests and furrows) structures, linking to each other vertically and thus attaching single cellular layers".

Hormonal state and surface pattern

During the menstrual cycle the thickness of the ectocervical epithelium and the glycogen content of the cells change. In the follicular phase, folded exfoliating cells are present on the epithelium. However, the relation between the surface pattern and the cyclical activity has not been investigated systematically. Allen et al. (1976) have examined 218 patients and found variability in cell surface structure between patients. Burgos and Roig de Vargas-Linares (1978) have found changing intercellular space in the epithelium during the menstrual cycle. However, in mice (Rubio, 1976) and rats (Parakkal, 1974) the cell surface pattern dependency on cyclical activity has been shown. In these animals microridges are only present on cells of an epithelium consisting of many cell layers. Such an epithelium is present during the estrous stage (Rubio, 1976, Parakkal, 1974) and in estrogen treated ovariectomized animals (Lamb et al., 1978). In humans the hormonal state dependency of the ectocervical cell surface structure expresses itself during pregnancy, the use of progestational agents (Ludwig and Metzger, 1976) and during postmenopause (Allen et al., 1976, Steger and Hafez, 1978). Ludwig and Metzger (1976) have found that during pregnancy the microridges are very prominent and can be arranged in small circles. The effect of progestational agents seems to be dose dependent. Whilst a low dose does not influence the surface pattern according to Moghissi and Reame (1976), a high dose causes crumbled surfaces with the appearance of variable microvilli and the disappearance of microridges, and cells which are separated from each other (Ludwig and Metzger, 1976). In postmenopausal women the number of microridges decreases, the microridges are less evident, their orientation is longitudinal (Steger and Hafez, 1978), and the cell border structures change from tender to coarse (Ludwig and Metzger, 1976). It appears from the postmenopausal effect that it is not easy to distinguish between the effect of hormonal state and age.

It is striking that microridges are distinctly present on cells of an epithelium which consists of many cell layers in states such as pregnancy and in estrus. Possibly, microridges represent the very last stage of cell differentiation, which does not occur in relatively thin epithelium with early desquamated cells.

Surface pattern and preparation procedures

Whereas it is well known that the SEM surface structure of free living cells, such as blood cells (Barber and Burkholder, 1975) or cultivated cells (Brunk et al., 1975), are very sensitive to

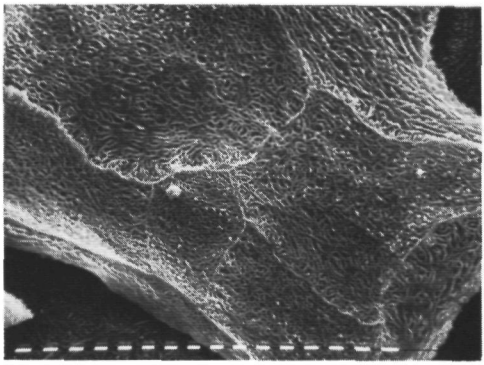


Fig. 1. Exfoliated superficial ectocervical cell; the surface of the flat polygonal cell predominantly shows microridges (bar = 1 µm).

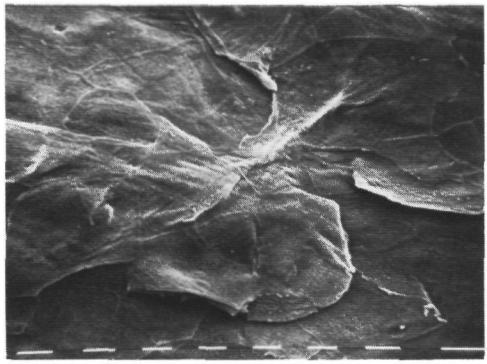


Fig. 2. The lumen bordering part of the ectocervical epithelium; the cells are arranged in an irregular pavement-like pattern; some cells are exfoliating (bar = 10 µm).

preparation procedures, this apparently does not apply to the superficial cells of the ectocervical epithelium. The following arguments may be put forward to illustrate the relative insensitivity of the SEM surface structure of normal superficial cells.

1) Different investigators have given identical or almost identical descriptions of the surface pattern of exfoliated cells (Murphy et al., 1973; Llanes et al., 1973; Leif et al., 1975; Kjaergaard and Poulsen, 1977) and exfoliating cells (Jordan and Williams, 1971; Nasr et al., 1973; Ferenczy and Richart, 1973; Bonilla-Musoles et al., 1974; Rubio and Einhorn, 1977; Sherman, 1977), albeit that the fixation procedures show differences. Moreover, the SEM image of exfoliated cells is not influenced by the cell collecting method and the washing procedure which precede cell fixation. Quite comparable pictures have been published after smearing the cells on a cover slip with a spatula (Murphy et al., 1973; Llanes et al., 1973; Bahr et al., 1976) or with a cotton swab (Kjaergaard and Poulsen, 1977), after sedimentation by centrifugation (Leif et al., 1975) or with the apparatus of Sayk (Kenemans et al., 1980), or after collecting the cells with a membrane filter (Murphy et al., 1973).

2) The SEM surface structural characteristics of exfoliating and exfoliated cells have been found to be about the same (Murphy et al., 1973; Williams et al., 1973a,b), which implies that the structures have not been changed by the process of exfoliation or the stay in the mostly acidic vaginal lumen.

3) Even after prior treatment of the exfoliated cells for routine light microscopic examination and classification, the SEM image does not deviate from normal (Murphy et al., 1973; Llanes et al., 1973; Allen et al., 1976; Bahr et al., 1976; Kenemans et al., 1980).

4) The surface pattern is still present on cells of the ectocervical epithelium which has been paraffin-embedded for LM study and then reprocessed for SEM (Carr et al., 1980).

5) We have purposely modified our routine cell preparation procedure to check the last but one point. Superficial cells were kept in a buffer for a maximum of ten days before fixation. They were collected on a Millipore® filter using a syringe. Subsequently, a glutaraldehyde fixative and a washing Na-cacodylate buffer were flushed through the syringe. After removing the syringe, the filter was placed upside down on a polylysine coated slide and placed in a humid atmosphere in a Petri dish. After removing the filter, the cells were stained for light microscopy. Subsequent SEM study revealed the cell surface pattern described previously. Apparently no alterations due to this preparation technique were visible.

Even the use of routine SEM fixatives is not necessary. When a formalin fixation or a freeze-fixation (with liquid nitrogen) followed by freeze drying is used, a similar structural pattern is seen as with glutaraldehyde (Williams et al., 1973b). Only when alcohol is used as a fixative (Friedlander, 1969; Llanes et al., 1973), an unusual SEM image can be seen. Air drying instead of critical point drying (Nasr et al., 1973; Williams et al., 1973a,b; Llanes et al., 1973) causes more shrinking, so bulging nuclei can be seen on the cell surface.

Our experiments seem to indicate that the non-superficial ectocervical cells are more sensitive to the effect of preparation procedures than are the superficial cells. It seems likely that this is true for preneoplastic and neoplastic cells as well. Friedlander (1969) has found that cells of carcinoma in situ are markedly distorted by alcohol fixation in contrast to normal superficial cells.

It is not clear why the surface structure of superficial cells is so stable. This is possibly related to the fully differentiated cytoskeleton.

Information in the literature is insufficient to determine the differentiation pattern of the surface structure of cells during migration from the basal to the superficial layer. Therefore, more systematic investigations are needed. Two

techniques are described below for such an examination.

Preparation of specimens

For our study of the surface pattern of cells from all layers of the human ectocervix, two SEM techniques have been applied: a stripping technique for the superficial cells, and a freeze-fracture technique for the intermediate, parabasal and basal cells. The results from these SEM studies have been compared with and completed by the results from TEM studies.

General SEM preparation

Biopsies from normal human uterine ectocervical epithelium, with an epithelial covering of about 0.5-1.0 cm^2, were taken after colposcopical examination. To remove adhering mucus and blood, the tissue blocks were washed in a 4% sucrose solution buffered with 0.1M cacodylate (pH 7.3). Thereafter the biopsy tissue was fixed in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) for at least 24 hours. Subsequently, the specimen was washed in the same buffer overnight and thereafter dehydrated in acetone (30, 50, 70, 90, 2x100%; 15 minutes each stage). After critical point drying using liquid CO_2, the specimen was mounted on a stub with silver paste and sputter-coated with gold (layer thickness about 30 nm). The specimens were examined in a Philips SEM 500 scanning electron microscope at an accelerating voltage of 25 kV.

Stripping technique

The stripping technique proceeded as follows. After SEM examination of the biopsy surface a strip of double adhesive tape (Sellotape®, 1.5 cm wide) was pressed slightly against the epithelium and then pulled off carefully (fig. 3). The tape was mounted on an aluminium specimen stub with the cells facing upwards. Using a stereo microscope (magnification 30x), landmark drawings were made of both the surface of the biopsy and the material on the tape. The location of landmarks was facilitated by the absence of the gold coating on the stripped area. Moreover, its irregular outline was very helpful when looking for matching points. After renewed sputter-coating the stripped biopsy and the cell material on tape were studied with the SEM using the drawings to locate corresponding points. The precise establishment of the cell surfaces originally contacting one another was done with the aid of the pattern of crests and furrows. The procedure coud be repeated several times on the same part of the biopsy (recognizable by the difference in thickness of the goldlayer coating). Only cells of the superficial zone could be stripped. After termination of the SEM examination the remaining biopsy tissue was prepared for light microscopic study as described by Ayres et al. (1971). For postscanning light microscopic study of the stripped cells, the pieces of tape were embedded in 4% agar and thereafter dehydrated in acetone, transferred in propylenoxide and embedded in epon. Thus it is possible to establish the number of cell layers present on each tape. For a detailed description of the stripping technique, the reader is referred to Davina et al. (1980).

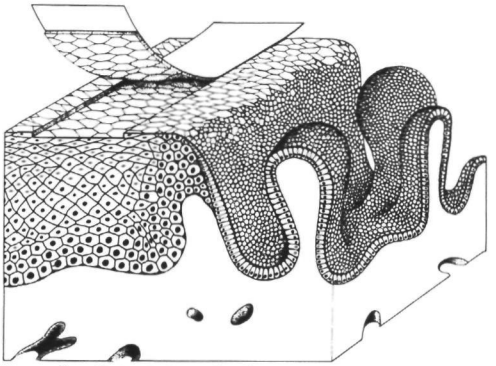


Fig. 3. Principle of the stripping technique; double adhesive tape is pressed slightly against the fixed, dehydrated and dried epithelium and pulled off carefully, removing one layer of partially overlying cells (modified after R. Cartier).

Freeze-fracture technique

Because the stripping technique was not suitable for cell layers beneath the superficial zone, a freeze-fracture technique has been applied for these layers. The technique of Humphreys et al. (1974) which has the advantage of preventing damage due to freezing, fracturing, thawing or critical point drying, was used with minor modifications. After dehydration of the fixed tissue in acetone, the tissue was transferred to a metal cup with liquid nitrogen. A small cooled chisel was used to fracture the epithelium in the liquid nitrogen. Subsequently, tissue fragments were transferred to acetone 100% and critical point dried.

Differentiation of the surface pattern of ectocervical epithelial cells

Results

In the normal ectocervical epithelium, between 20 and 25 cell layers are present, divided into four zones (Jordan and Singer, 1976). While migrating from the basal layer to the lumen, the cell shape changes considerably. The basal zone is composed of only one layer of cuboïd to cylindrical cells. The 3 to 5 cell layer thick parabasal zone consists of polyhedral cells. The intermediate zone possesses 8 to 10 cell layers of large, round or somewhat flattened cells. The superficial zone consists of 7 to 12 cell layers of flattened, partially exfoliating cells (fig.4).

Transmission electron microscopic (TEM) studies of the ectocervical epithelium are relatively few. They have been carried out by Ashworth et al. (1960), Hackeman et al. (1968), Shingleton et al. (1968), Ferenczy and Richart (1974) and Dembitzer et al. (1976). From these studies it is known that the cells of the basal and parabasal zone bear many villus-like processes which possess desmosomes at the top. In this way cell-to-cell contacts are formed, which leave a large interlacing

intercellular space. This space becomes smaller in the intermediate zone of the epithelium. The number of intact desmosomes and gap junctions in the basal, parabasal and intermediate zones is large, whereas they are hardly present or absent in the superficial zone (McNutt et al., 1971; Wiernik et al., 1973; Ferenczy and Gelfand, 1979). In the superficial zone only a very narrow intercellular space is present. Here the plasma membranes show a fine undulating pattern and neighbouring cells are interdigitated in a zip-fastener fashion. In the basal and parabasal cells the orientation of cytoskeletal elements which are connected with the plasma membrane is approximately perpendicular to the membrane. In the intermediate and superficial zones on the contrary, they are arranged more parallel to the cell surface.

The stripping technique clearly reveals differences in the SEM surface structure of the upper (or lumen facing) and lower (or basal lamina facing) side of all superficial cells. The luminal side of the cells characteristically shows microridges and crests (fig. 5a), whereas the basal side is studded with numerous globular microvilli and furrows (fig. 5b). Microridges and globular microvilli as well as crests and furrows are interdigitating structures. Also on the luminal side some microvilli are present between the microridges, but on the basal side microridges do not occur. The microridges vary in length from 0.5 to 5.0 µm and in width from 0.10 to 0.25 µm. Further, the orientation and the degree of branching and anastomosing is variable. The globular microvilli vary in top diameter from 0.15 to 0.25 µm; when their base on the surface has somewhat been extended in length, the structures are called microplicae. Crests and furrows delineate regions of variable size. On exfoliated cells it is seen that they occur not only at the cell margins, but also more centrally on the cell surface (fig. 1 and 4).

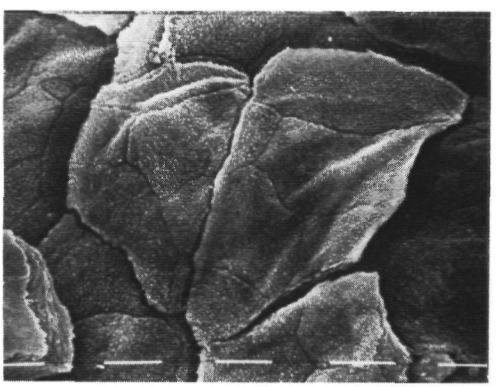


Fig. 4. Basal side of exfoliated superficial ectocervical cells showing globular microvilli and furrows; furrows occur on the cell surface and do not delineate cell borders (bar = 10 µm).

The surface structural pattern varies with the location of the cell in the superficial zone. During migration from the deepest lying layer to the lumen bordering layer of the superficial zone, the crests and furrows become more prominent; moreover, the degree of branching and anastomosing of the microridges increases as well as the width of the microridges (from 0.10 to 0.25 µm) and the top diameter of the microvilli (from 0.15 to 0.25 µm) (compare fig. 5 and 6). The space between microridges and microvilli decreases. The images seem to indicate that the number of microridges and microvilli per area does not change during cell migration in the superficial zone. The pattern formed by microridges and microvilli varies. This variation exists between the regions outlined by crests and furrows, so the surface of a cell can show "regional" differences (fig. 7).

Compared with the stripping technique, freeze-fracturing possesses the disadvantage that it cannot be applied systematically and in a particular direction. Therefore, it is necessary to determine precisely how the fracture plane occurs. It is also disadvantageous for the examination of the cell surface that the fracture planes do not occur exclusively between the cells, but also occur intracellularly. However, intercellular fracture planes can be seen relatively frequently because of the large size of the intercellular space. The cell surface pattern of the intermediate zone shows filiform processes and some globular microvilli (fig. 8). The filiform processes probably represent the intercellular connections with desmosomes at their top as seen in the TEM. Intermediate cells located near the superficial zone mainly reveal globular microvilli and only few microridges; crests or furrows cannot be distinguished (fig. 9). The parabasal cell surface possesses more or less foliate processes (fig. 10); here, the intercellular space is larger than it is in the intermediate zone (about 1.5 and 1.0 µm respectively). The processes on the basal cell surface show a more pronounced foliate character compared with the parabasal cell processes (fig. 11). Underneath the basal cells, the basal membrane and the connective tissue can be seen.

Summarizing the results from our stripping and freeze-fracturing studies, the following can be said about the differentiation of the surface pattern of cells migrating from the basal lamina to the lumen of the ectocervix.

1) The cell shape changes from cuboïdal to cylindrical in the basal, to round in the parabasal and intermediate zone, to flattened in the superficial zone. Moreover, the volume of the intermediate cells has increased when compared with the parabasal cells.

2) The size of the intercellular space is changing. It is relatively large in the basal and parabasal zones, and small or virtually absent in the intermediate and superficial zones, respectively.

3) The processes of the cells change their character. In the basal, parabasal and intermediate zone the processes appear to change form foliate to filiform. They are probably structures by which neighbouring cells are connected to each other. Additionally, the processes are shorter in the

intermediate zone. They are shortest on the cells in the superficial zone It is unlikely that the processes of the intermediate cells differentiate into globular microvilli and microridges of the superficial cells.

4) The microridges and crests on the luminal side, and the globular microvilli and furrows on the basal side are specific for the superficial cells. These structures are most prominently developed in the upper part of the superficial zone They possibly increase the surface adhesiveness by interdigitating, in addition to the diminished desmosomal attachment.

Discussion

There is a paucity of data in the literature about the differentiation of the cell surface pattern of normal human ectocervical epithelial cells. Llanes et al. (1973) have concluded from studies on exfoliated cells that basal and parabasal cells possess finer and longer microvilli than those of the intermediate cells, whereas the superficial cells possess low microridge structures The deviant surface structure of basal and parabasal cells as compared with our results can be related to the exfoliated stage or the alcohol fixation of these cells. Fink and Thomas (1975) studied ectocervical epithelium in organ culture. Due to the progressive loss of the superficial and later also of the intermediate and parabasal cells, the surface architecture of the various layers could be studied. The less mature cells showed incomplete and less closely packed microridges, whereas the basal cells displayed a markedly different surface structure to the parabasal, intermediate and superficial cells. It seems unlikely, however, that the results from such studies can be extrapolated to the normal in vivo situation. From studies on metaplastic cells in the squamo-columnar junction, Ferenczy and Richart (1973) and Williams et al. (1973a,b) concluded that microridges develop in a very late stage of maturation of the squamous epithelium.

The fact that the luminal side of superficial cells shows a surface pattern other than that of the basal side has long been undetected. The view has existed that microridges cover both the upper and lower surface of superficial cells (Ferenczy, 1980). Even in smears, where both sides can be examined, the difference between both cell sides has not been noticed. Murphy et al. (1973) have compared the SEM images of exfoliated cells with those of cells detached from the epithelium by pressing a membrane filter on it. The deviant surface pattern of the latter group (i.e of the basal side of the cells) was interpreted as being an artifact due to the cell collecting method. Domagala et al. (1979), studying exfoliated bladder cells, established the ratio of cells bearing microridges and microvillus bearing cells. From their findings they did not deduce that the upper and lower side differ in structure.

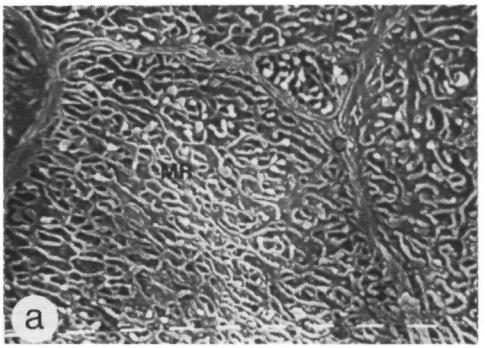
Rubio(1976) studying mice treated with estrogens, was the first to observe differences between luminal and basal cell surface structure. Kenemans et al. (1980) were the first to describe differences between the luminal and the basal surface of superficial cells of the human ectocervix. Their conclusions were based on the following observations a) folded superficial cells showed two different surface patterns, b) by comparison of the number of exfoliated superficial cells, about half of the cells showed microridges on their surface and half possessed microvilli, c) examination of adjoining cell surfaces in cleaved tissue revealed different surface structures.

Crests and furrows are also called bars and grooves, respectively, they have often been considered to be cell borders (Jordan and Williams, 1971, Williams et al., 1973a,b, Bonilla-Musoles et al , 1974, Fink and Thomas, 1975, Rubio and Kranz, 1976, Sherman, 1977, Lamb et al., 1978, Steger and Hafez, 1978). However, this study has shown that crests and furrows are not only border structures, but are also impressions on a cell surface from borders of two or more neighbouring cells which have lain above or beneath. Murphy et al (1975), Bahr et al. (1976) and Ludwig and Metzger (1976) have interpreted them in this way, but they did not distinghuish that crests are present on the luminal side and furrows on the basal side. In our view, the crests and furrows develop as follows. When flattening during migration upwards, cells from one particular layer press against each other sideways. Their lateral cell margins are pushed upwards. As a consequence, the elevating cell borders form crests at the luminal side and furrows at the basal side. Crests induce "secondary" furrows in the cells lying above, whereas the furrows induce "secondary" crests on the cells lying beneath. On exfoliated cells the secondary crests and furrows are most distinct.

It is striking that superficial cells from various non-keratinizing stratified epithelia develop microridges. This holds true for the epithelium of the ectocervix, the bladder (Hodges et al., 1977, Hodges, 1978, Tannenbaum et al., 1978, Hodges, 1979), the oral mucosa (Parakkal, 1974), the oesophagus (Logan et al., 1977) and the cornea (Blumcke and Morgenroth, 1967). Also, in the stratum corneum of the skin (Odland and Reed, 1967, Matoltsy and Parakkal, 1967) and in fish epidermis (Schliwa, 1975), microridges are present on the cells. In the fish epidermal cells the development of surface protrusions appear to be related to the modifications of the cytoskeleton (Bereiter-Hahn et al., 1979).

It is tempting to hypothesize on functional significance of the surface architecture of the superficial cells. Several functions have been proposed for example a better fixation of mucus on the luminal surface (Sperry and Wassersug, 1976, Ludwig and Metzger, 1976), mucus transport (Ludwig and Metzger, 1976), and an optimal maintenance of the epithelial integrity by increasing the area of contact between cells offering resistance to sideways movements (Williams et al., 1973a,b, Ferenczy and Richart, 1973).

It is not clear why ridges are hardly present or absent on ectocervical superficial cells during postmenopause (Steger and Hafez, 1978), during particular stages of the estrous cycle (Parakkal, 1974, Rubio, 1976), after ovariectomy (Parakkal, 1974, Lamb, 1978) or during (pre)neoplastic transformation (Williams et al., 1973a,b, Ferenczy and Richart, 1973). Microridges may be an expression of the estrogen effect. It seems justified to conclude from these date that a changing hormonal



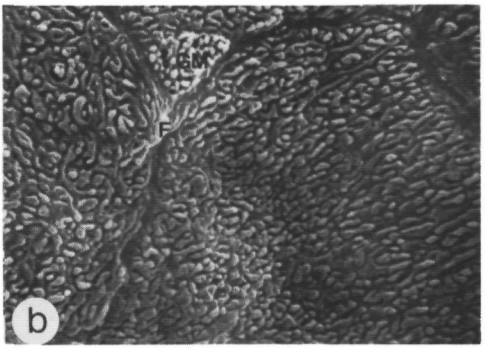
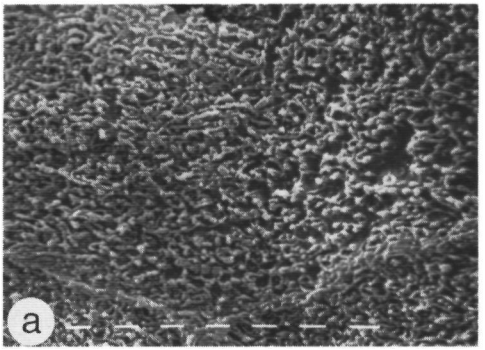


Fig. 5. Adjoining cell surfaces of the superficial zone of the ectocervical epithelium after the sixth stripping; fig. 5a shows the luminal surfaces of cells in the biopsy tissue with microridges (MR) and crests (C); fig. 5b shows the basal surfaces of the cells on the tape with globular microvilli (GM) and furrows (F) (bar = 1 µm).



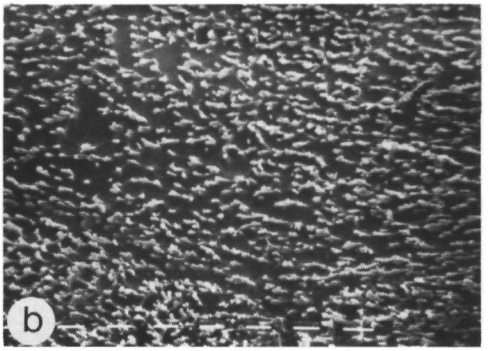
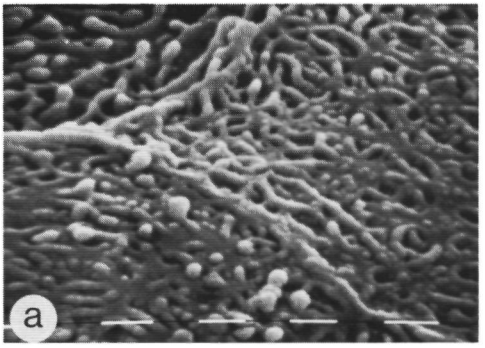


Fig. 6. Adjoining cell surfaces of the superficial zone of the ectocervical epithelium after the tenth stripping; fig. 6a shows the luminal surfaces, fig. 6b shows the basal surfaces; compared with fig. 5 the microridges are less wide and the top diameter of the microvillus is smaller, whereas the crests and furrows are less prominent (bar = 1 µm).



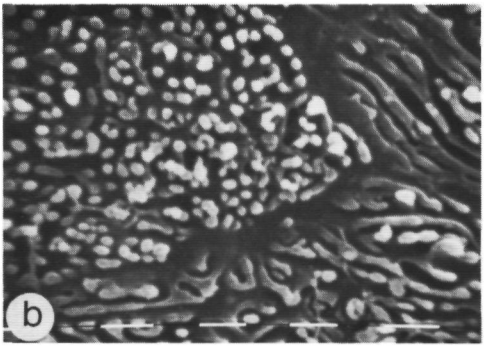


Fig. 7. Adjoining cell surfaces of the superficial zone of the ectocervical epithelium after the second stripping; fig. 7a shows the luminal surface, fig. 7b shows the basal surface; the variation in microridge- and microvillus-pattern is shown, which is related to the regions outlined by crests and furrows (bar = 1 µm).

state or the induction of a modified cell cycle causes an aberrant course of differentiation

Concluding remarks

Microridges and crests are only present on the luminal side of superficial cells The basal side possesses only globular microvilli and furrows. These structures are developed in the superficial zone and are most prominent in the upper part of this zone. The occurence of microridges in the superficial zone only, and the absence or poor development during certain hormonal states and abnormal differentiation, shows that microridges are only present on well-developed cells

The existence of regional structural differences on superficial cells demonstrates that the surface pattern is not only determined by the cell itself, but is also the result of mutual influences of adjoining cells.

Our study of the surface pattern differentiation of the epithelial cells of the human ectocervix reveals that there are gradually changing surface structures on the cells in the four zones. Cells of the basal, parabasal and intermediate zones show progressively shorter and finer cellular processes Parallel with this shortening, the size of the intercellular space decreases. In the superficial zone, interdigitating globular microvilli and microridges, as well as furrows and crests which leave only very little intercellular space, are present The type of surface structure probably reflects functional differences foliate or filiform processes equipped with desmosomes for enhancing of intercellular connections and metabolic exchanges to the extracellular environment, and interdigitating structures with hardly any desmosomes for increasing cell surface adhesiveness and resisting sideways movements

Acknowledgements

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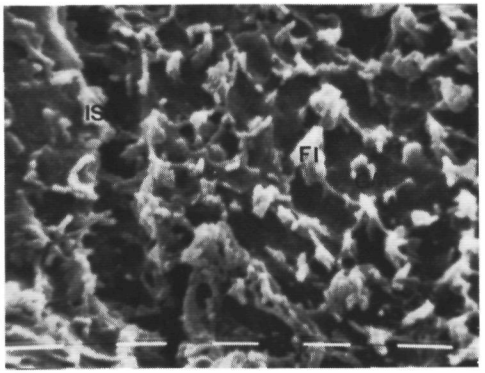

Fig. 8. Epithelial cell in the intermediate zone after freeze-fracturing; the surface possesses filiform processes (FI) and some globular microvilli (GM); the intercellular space (IS) is relatively narrow (bar = 1 μm).

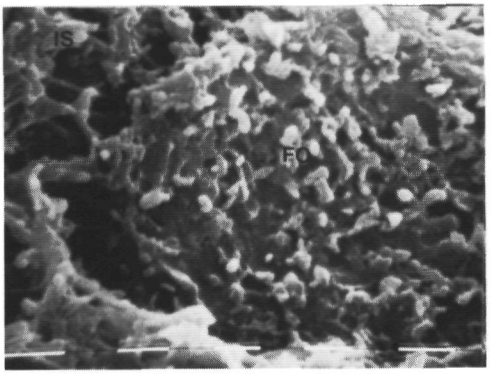

Fig. 10. Epithelial cell in the parabasal zone after freeze-fracturing; the surface shows more or less foliate processes (FO); the intercellular space (IS) is relatively large (bar = 1 μm).

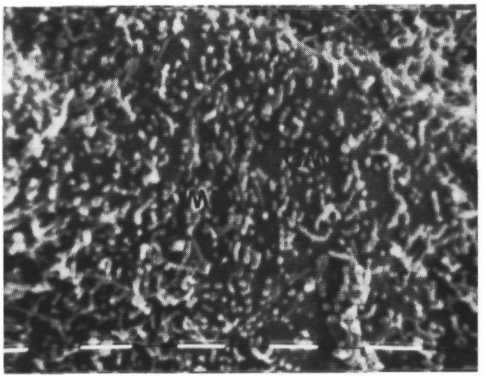

Fig. 9. Intermediate cell near the superficial zone after freeze-fracturing; the surface mainly reveals globular microvilli (GM) and only few microridges (MR) (bar = 1 μm).

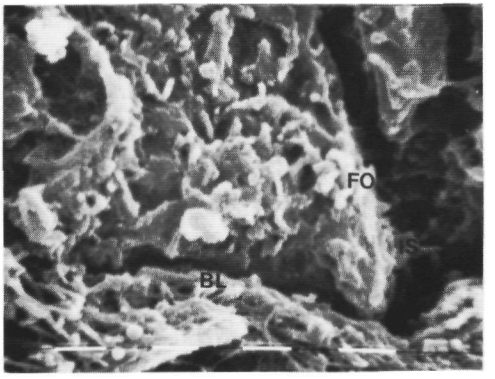

Fig. 11. Epithelial cell in the basal zone after freeze-fracturing; the surface possesses foliate processes (FO); the intercellular space (IS) and the basal lamina (BL) are shown (bar = 1 μm).

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Discussion with reviewers

H. Ludwig Under the section of preparation of specimens the authors state that "The procedure (stripping) could be repeated several times on the same part of the biopsy . .". How many times was stripping usually performed on a given tissue location to remove the entire superficial zone?

R.W. Steger Can you show light microscopical cross sections of the tissue after the "stripping" procedures?

Authors It usually requires 7 to 12 strippings to remove the entire superficial zone Post scanning light microscopical study of biopsy tissues revealed that the superficial zone around the stripping place consisted of 7 to 12 cell layers, what correspond with the number of performed strippings (fig. 12).

G.M Hodges Why is it possible to strip only cells of the superficial epithelial layer?

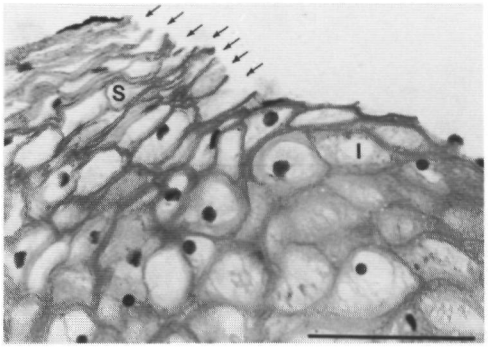

Fig. 12. Light micrograph of the ectocervical epithelium which has been stripped seven times and studied in SEM; seven superficial cell layers (s) had been removed; the last cleavage plane occurs within the cytoplasm of the intermediate cells (i) (bar = 100 µm).

Authors: It is likely that stripping of superficial cells only, is related to the change in desmosome structure, especially the break up of desmosomes. Information about the desmosomes in different zones of the ectocervical epithelium is scarce. However, it appeared in the epidermis, that desmosomes abruptly change at the transition site of stratum granulosum and stratum corneum (text reference Odland and Reed, 1967), whereas the complete stratum corneum can be stripped (Pinkus H, Examination of the epidermis by the strip method of removing horny layers, J. Invest. Derm. 16. 1951. 383-386).

H. Ludwig: The new stripping technique allows deeper levels to be viewed by removing the more superficial layers. Can the authors rule out, however, that the resulting surface does not contain artifacts due to the physical manipulation of overlying structures? Figures 1-6 present no problem, but are the authors sure that their techniques do not produce artifacts in samples of deeper layers, as represented in figures 8-11?
G.M. Hodges: Could you expand on your freeze-fracture procedure?
Authors: Artifacts can not be produced in samples of deeper layers (fig. 8-11) due to previous stripping, because freeze-fracturing was performed on unstripped specimens.

H. Ludwig: The authors state that the surfaces shown in figure 8 and 10, are of intermediate cells and parabasal cells, respectively. TEM and LM evidence for this is missing from the manuscript. Could the authors at least add one TEM or one LM to offer material for parallel comparison?
Authors: No TEM or LM study has been performed after SEM study of freeze-fracture specimens.

L.G. Koss: How was the increase in volume of cells determined?
Authors: The volume of cells in different epithelial zones has not been measured actually. But our TEM and LM studies revealed that the diameter of cells and the width of the intercellular space in the parabasal zone distinctly and consistently differ from those in the intermediate zone.

U. Brunk: What are your reasons to believe that the "filiform processes" of fig. 8 correspond to desmosomes?
Authors: The filiform processes are the sites where neighbouring cells are connected by desmosomes as can be seen in TEM. When the freeze-fracture plane occurs intercellularly, the filiform processes are broken near the desmosomal attachment.

G.M. Hodges: The superficial epithelial cells of the ectocervix appear relatively insensitive to different preparative procedures: can this be correlated to the presence of intermediatesized filaments of the prekeratin type; to what extent do such filaments vary within cells of the different epithelial layers and could you define the characteristics of the fully-differentiated cytoskeleton of superficial cells?
Authors: Data on the cytoskeleton of ectocervical cells are very scarce. It may be that the relative insensitivity of the surface of superficial cells is related to the location, density and chemical composition of the intermediate filaments, which change during migration of the cells upwards. The fully-differentiated cytoskeleton of superficial cells consists of many interwoven tonofilaments concentrated and sometimes aggregated near the cell membrane.

H. Ludwig: The authors performed post scanning light microscopic studies of every specimen investigated. The results of these studies, if normal might in part prove that no artifacts were present in the SEM samples. However, the authors do not specify the way in which post scanning specimens were sectioned. In other words, depending on the angle of cut, is it not possible that previous artificial disruption of the tissue, if present, would not be seen?
Authors: For post scanning light microscopic study of the stripped layers and the remaining biopsy tissue, the specimens were cut perpendicularly to the flattened cells. Thus, the SEM surface could be studied extensively in the light microscope. Although we cannot exclude the ignorance of previous artificial disruption, this is very unlikely.

H. Ludwig: Critical point drying is effective for drying all layers, but only preserves structures on the surface. The authors use freeze-fracture to separate layers deeper than the superficial zone. Are they sure that the structures seen in micrographs of these so treated deeper layers are the original structures (i.e. figure 8)?
Authors: The tissue specimens have been freeze-fractured before critical point drying.

Reviewer I: How long did you wash and fix the biopsy specimens and what was the osmolality and temperature of the buffer and fixative?

Authors The biopsy specimens were washed in (.1 M cacody ate buffer (pH 7.3, 320 mOsm , 20° ( ) for about 15 minutes The fixat on in buffered 2% glutaraldehyde (pH 7.3, 550 mOsm., 4° C) last·d at least 24 hours Subsequently, the same cacody-late buffer was used for washing the fixed specimens.

L.G. Koss Are the authors sure that the number of desmosomes is reduced in the superficia' layers of the squamous epitheliur? A reliab e statist.cal study has not been done. It is more likely that, as this epithelium matures, the desmosomes break up, which accounts for the ease with which the superficial layer can be lifted (see Koss L G , Diagnostic Cytology, Ed. 3, Lippincott, Philadelphia, USA, 1979, p 160 and fig. 8-4)

Authors We have seen repeatedly, that the desmosomes in the superficial zone show alterations and ruptures, even so that only a thickening of the cell membrane can be seen. This may account for the observed reduction in number of intact desmosomes compared with the number in the intermediate zone. Desides, we established that the desmosomes of superficial cells are smaller than those of intermediate cells These observations have been taken earlier by Burgos and Roig de Vargas-Linares (text reference).

Chapter 2 **Cell Surface Morphology in Epithelial Malignancy and its Precursor Lesions**

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Abstract

The cell surface organization of cancer cells is of potentially great significance, as it may not only allow (early) diagnosis, but as it may also harbour markers for refined prognosis (degree of oncogenetic and metastatic potential), and targets for selective cancer (chemo- and immuno) therapy With these aspects in mind, the present review deals with SEM work done on (pre-) malignant cells, both in vivo and in vitro, and in animal models Attention, however, is focused on human cancer cells.

Cancer cells in vitro may lose many of their original malignant characteristics, and show adaptations to culture conditions Many other factors have been shown to influence cell surface morphology, such as cell cycle, cell contacts,and preparation technique

Cancer cells differ in their surface morphology from normal cells, and have an extra ordinary amount of surface activity Human malignant epithelial cells show abundant long, pleomorphic microvilli, especially those present in effusions In squamous epithelium (bladder, cervix) microridge systems present on normal superficial cells are progressively replaced by microvilli which increase in number and degree of pleomorphism during experimental and clinical oncogenesis.

The question of whether or not the appearance of long, pleomorphic microvilli reflects an irreversible alteration of the epithelium, and thus provides an early marker of irreversible neoplastic transformation is considered and assessed on the basis of our own work with (pre-) malignant cells of the human uterine cervix

Although SEM has contributed significantly to the description of oncogenesis,up to now it has no early diagnostic, prognostic or therapeutic significance.

KEY WORDS. microvilli, microridges, cancer cells, neoplastic cells, malignant cells, malignancy, premalignancy, carcinogenesis, cervix uteri, epithelium.

Introduction

Cancer can be described in terms of defective cell-to-cell interactions on the basis of alterations in the cell periphery

Phenotypical transformations within the plasma membrane-cell surface complex, although secondary to genetic changes associated with oncogenesis,are early events in a process which *results ultimately in asocial behavior of cells.* Characteristically, this aberrant behavior shows an escape from normal growth and positional controls These control functions, i e , inhibition of cell replication and inhibition of cell motility, respectively, have been shown to be cell contact-dependent phenomena, closely related to a normal cell surface organization of the interacting neighbouring cells Thus, these in vitro phenomena, and their in vivo equivalents, (e g , unrestrained proliferation, invasive migration and metastatic growth), have been claimed to be related to changes in surface properties of the neoplastic cell [1-5]

Theoretically, the cell surface organization on cancer cells is of paramount importance, as it may not only allow (early) diagnosis, but it may also harbour markers for refined prognosis (degree of oncogenetic and metastatic potential), and targets for selective cancer (chemo- and immuno-) therapy It should be borne in mind that the present review, dealing with SEM work on (pre-) malignant cells, the cell surface organization mentioned above, is necessarily reduced to those structural aspects which can be studied with SEM in the Secondary Electron mode (S.E.). Attention is focused on human cancer cells. Furthermore, a specific assessment is made of the usefulness of cell surface ultrastructural characteristics as early markers of irreversible neoplastic transformation, treating the prognostic and therapeutic aspects only in passing.

SEM studies of Cancer Cells

Observations on the surface morphology of cancer cells have been made by using a number of methods, e g , phase contrast cine-light microscopy, conventional transmission electron microscopy (cTEM), TEM replica studies, Freeze Etch techniques and scanning electron microscopy (SEM) [6-8] Until now, TEM and SEM have had the drawback

SCANNING ELECTRON MICROSCOPY/1981/III (Pages 23 36). SEM Inc., AMF O'Hare (Chicago), IL 60666, USA

of the inability to study living cells, thus losing the 'dynamic temporal aspect of cell morphology'[9]. Normally, it only depicts the outermost part of the cell surface complex. Moreover, SEM needs (preparation) techniques for image forming, which influence the cell topography. The advantage of SEM is obvious: large parts of cell surface can be studied at various magnifications with great depth of field.

This always allows correlations between local ultra structures with the overall organization of the cells or tissue of interest, in statistically meaningful numbers of cells within a population.

The "cancer cells have been studied under a large variety of conditions: dead or alive, of epithelial origin or of non epithelial origin, obtained in vivo or in vitro, either artificially transformed or from (primary or metastatic) tumor sites, cultured for a long or short period and studied by researchers using different techniques. The cells, of human or animal origin, have been processed by different methods either within a suspension of exfoliated, free-floating or trypsinized cells, or within a tissue. As so many factors are involved it seems unlikely that something such as 'the cell surface morphology' of 'the cancer cell' will become evident. On the other hand the possibility that topographic features can reliably denote specific cancer cells in SEM-images is too intriguing to neglect, and indeed many authors have tried to validate the assumption that it really does.

Using a TEM carbon replica method, Easty and Mercier (1960) were among the first to describe morphological differences, viz., in the density of long microvilli, between normal cells and their neoplastic counterparts[10]. The first detailed SEM work on cancer cells came some ten years later[11-13]. Critical reviews[6-7] of the literature up to 1973 showed the following. First, although there was a wide variety both within and between cell populations, many tumor cells had in common an 'extraordinary amount of surface activity" (microvilli, blebs, ruffles and other lamellipodia)[6], seen in normal cells only in mitosis[6,14], *activity which may be interpreted as a specialization of the neoplastic cell surface for macropinocytosis*[7].

Secondly, most of the work had been done on cultured normal cells or cells transformed either 'spontaneously', or by chemical, physical or viral factors. These cultivation studies posed the question, still current, of how to distinguish cell surface adaptations to (long term) culture conditions and neoplastic characteristics[7]. Moreover, cells in monolayer cultures appear to lose rapidly many of their original malignant properties[8].

And finally, as the majority of cells used have been fibroblasts, the majority of studies have been on (mesenchymal) sarcogenesis, rather than on (epithelial) carcinogenesis. Fibroblastic cell lines tend to have fewer microvillous processes, than that of epithelial lines[7,8]. Cell lines derived from human epithelial tumors e.g., Hela (cervix), HEp-2 (larynx) and KB (oral carcinoma), as studied extensively with TEM, and also with SEM (see[8]), show abundant long microvilli (density: 13 microvilli per square micrometer in Hela cells[15]), next to other surface micro extensions. The latter take the form of cytoplasmatic blebs in KB[16] and HEp-2[12,17] cells. These cell line studies did not show any morphological characteristic unique to the transformed state. In their extensive review of the ultrastructure of human tumor cells in vitro Seman and Dmochowski[8] concluded that these studies "have not revealed so far any specific morphological markers indicative of malignancy".

However, many transformed cells appear to have at least one feature in common, in comparison with their normal counterparts, viz., an increase in the number and density of (mostly long slender) microvilli: the "hairiness" of the cell surface.

In 1976 Allen et al[18] found that there was a 3-to-5-fold increase of rather short surface microvilli per unit area on non-dividing malignant rat liver epithelial cells in culture, compared to the normal cells under identical culture conditions. Others have obtained similar results with human neoplastic cells cultured in vitro[19-21] or after in vitro transformation either spontaneously or virally[22], chemically[23] or by X-irradiation[24]. Saxholm and Reith[23] were the first to point out the distinction between short and long (i.e., the length being more than three times the diameter) microvilli. They found that the concentration of short microvilli was a function of passage in culture, whereas the concentration of long microvilli correlated in a statistically significant way with the oncogenic potential of the cells. However others[25], also using fibroblasts, could find no statistically significant differences in densities of microvilli between non neoplastic and neoplastic lines of common origin. In recent reviews dealing with human epithelial cancer cells[26-30] attention is given to this important question regarding the diagnostic significance of microvilli on the cell surface.

Surfaces of free-floating, viable cells of metastatic human carcinomas, found in pleural and peritoneal fluids, were shown to be totally and densely covered by (rather long) microvilli of *variable configuration and distribution*[27,31-35], regardless of histological type of primary tumor origin. A similar microvilli dominated pattern was found on the surface of exfoliated abnormal cells[36,37], although not always on all abnormal cells[38].

Pleomorphic microvilli have also been demonstrated by SEM on free (luminal) surfaces of malignant human epithelial cells in situ, both in the adenocarcinoma-type (e.g., endometrium[39-40], gallbladder[41]) and in the (poorly differentiated) squamous cell carcinoma-type (e.g., bladder[42-46], cervix[47-53]).

Normal epithelial cells of the glandular type mostly display a microvillous pattern on their free surfaces. However, in glandular neoplasia (endometrium[39], breast[54]) a gradual decrease in the number and length of surface microvilli was noted. Thus, there seems to be a contrast, unexplained until now, between cancer cells in the tissue, which are in some instances almost devoid of microvilli, and the mostly, abundantly covered cancer cells in suspensions obtained from effusions or cell cultures. This

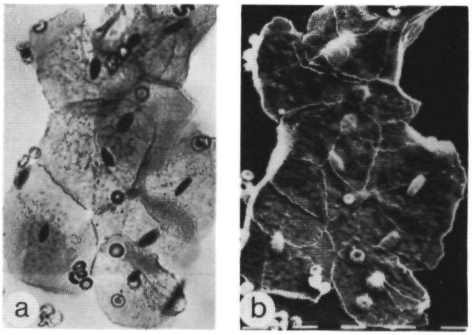
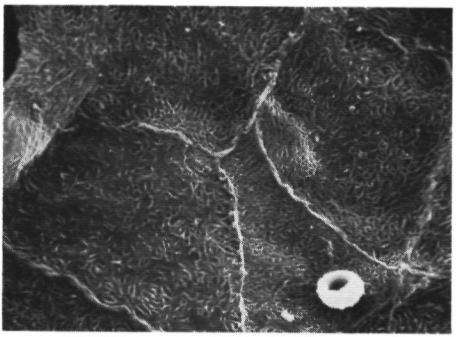
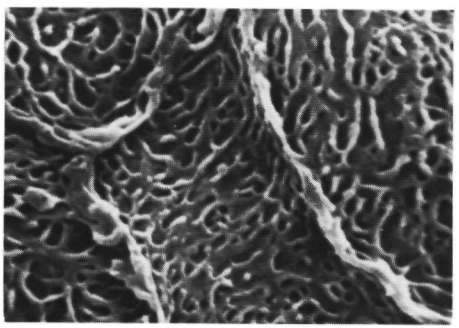


Fig. 1:
Consecutive observations on a sheet of normal, superficial and intermediate ectocervical cells by LM (a) and SEM (b). The technique permits identification in SEM of LM classified cells. (Bar = 10 μm).





Figs 2 and 3:
Higher magnification SEM micrographs of one of the cells situated in the sheet of cells of fig. 1, showing the characteristic surface micromorphology at the free, luminal side of the flat, polygonal cells of the human ectocervix, viz., a system of microridges, and prominent linear and terminal bars. (Bar = 1 μm).

difference was also noted by Hallowes and Peachy, who suggested assessing the influence that the surface of the culture (glass or plastic versus collagen gel) may have upon the topography of cells in cultures [55]. The study of Russo et al [56] on a (metastatic) human breast carcinoma cell line, revealing different cell surface architectures between cells with different growth patterns (either single cells, clusters or confluent monolayers) is also of interest in relation to this question, as it points to the possible modifications of cell surfaces by the interaction with neighbouring cell surfaces, surrounding medium or culture substrate. Moreover, in our opinion there is the possibility that the differences between cancer cells in situ and cancer cells in effusions could be explained partially in terms of differences between invasive cells of the primary site and metastatic cells obtained in body fluids.

Normal epithelial cells of the squamous (e.g. cervix, vagina, oral cavity, skin, bladder) have no microvillous free surface, but show a microplicae- or microridge-like type of surface architecture on the superficial cells, with small variations in design depending on cell type and degree of cell maturation. As squamous epithelial cells do display a distinct qualitative difference between normal ('microridge') cells and malignant ('microvilli') cells, the squamous epithelium is of special interest for the study of transformation of normal cells to cancer cells. (see for normal ectocervical cells fig. 1-3)

SEM studies of Precancerous Cells

Both human bladder urothelium [44] and ectocervical epithelium [57] have been claimed to offer a unique opportunity to study early epithelial malignancy in the human, and indeed they are unique in several respects, viz.,

1. The distinct, qualitative differences in cell surface architecture mentioned above, giving "specific ultrastructural markers of which the destiny could be followed during malignant transformation" [58].
2. The slow, well documented progression from normal cells to invasive cancer cells.
3. The accessibility of the involved tissue for sampling of cells and tissue and for optical monitoring of the process (cystoscopy/colposcopy).
4. The clinical demand to tolerate the lesions, and to spare the organ for as long as possible without danger to life (especially the bladder).
5. The availability of an accepted Light Microscope (LM) subclassification system, which can be used as a frame of reference for EM observations (especially the cervix).

The cervix [59], and especially the bladder [45, 58, 60] take a prominent position, not only in the study of human carcinogenesis, but also amongst the animal-model studies of experimental epithelial carcinoma. In experimental bladder cancer the normal microridge system on the luminal side of the superficial cells is progressively replaced by microvilli which increase in number and degree of pleomorphism during the experiment. Close analogies were found to exist between human

urothelium in vivo and rat urothelium in the animal model, both between pre-malignant lesions and between the cancerous lesions [45, 58, 60]. It was suggested that the appearance of pleomorphic microvilli may reflect an irreversible alteration of the bladder mucosa [60] and thus could "provide a convenient marker of irreversible neoplastic transformation" [45].

A similar gradual transformation of cell surface morphology was found in the premalignant range (i.e., dysplasia, Carcinoma in situ [CIS]) of human ectocervical epithelium [36,47-53,61,62]. This transformation, roughly, has as a result closely packed microvilli on small CIS cells replacing the prominent microridge system on the surface of the normal flat, large polygonal superficial cells. These unanimous reports have also provoked a tendency to reverse the relationship by hypothesising that cell surface microridges indicate normality, while cell surface microvilli imply abnormality.

The concept of premalignancy, or precancerous lesions is of special interest both to the clinician and the researcher. This concept is best worked out for squamous carcinoma of the cervix, both with LM [63], and with Freeze-etch techniques [64, 65] and TEM [66]. As TEM cannot distinguish microvilli cut longitudinally and microridges cut transversely [41, 53, 62] mistakes have been made with TEM interpreting microridges as microvilli.

SEM offers a more powerful approach to diagnostic cytopathology. The SEM approach would be of paramount importance if any of the ultrastructural cell surface parameters could be considered as an early marker of irreversible neoplastic transformation, as suggested by some authors [36, 37, 45, 57]. In order to investigate the significance of the surface ultrastructure of cells of the uterine cervix for the early detection of irreversible neoplastic transformation, we studied normal and abnormal ectocervical cells, both in exfoliated cell preparation and in tissue biopsies.

Material and Methods

a Cells

Normal and abnormal exfoliated and exfoliating cervical cells were obtained from patients selected on the basis of one or more preceding (and independently made) routine cytological diagnosis, using the Papanicolaou subclassification system, with a supplementary refined descriptive diagnosis. Patients were then colposcopically evaluated regarding location and character of their cervical lesion. Data on hormone status and cycle were noted. After photodocumentation of the lesion, cells were obtained by scraping the cervix with a cotton swab from the neoplastic site.

Cells were suspended in a 0.1 M cacodylate buffer and sedimented on polylysine coated pieces of glass. Half of the material was processed for LM (Pap-stain), while the other half was processed for SEM. Directly after sedimentation the cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (with sucrose, effective osmolarity 320 mOsm/L, pH 7.2, 37° C.) for ten minutes. Cells were examined with a PSEM 500 after washing, dehydration, critical point drying and sputter coating with gold-palladium.

In the second phase of the study a method for consecutive observations on the same cell by LM and SEM was applied. Cells, suspended as before in a buffer, were now sedimented on specially engraved (polylysine-coated) pieces of glass, and after fixation (also as before) stained according to Papanicolaou, and photomicrographed. After removal of the cover glass with xylol, cells were processed for SEM as described before.

b. Tissue

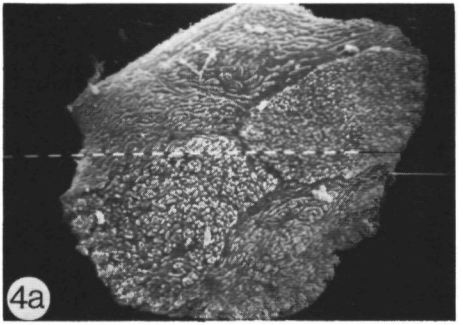
Tissue blocs were obtained from the same patients the cells had been obtained from, routinely, after several weeks allowing the lesion to re-epithelialize. Material was obtained either by colposcopically directed biopsies or by cone biopsies. Half of the tissue blocs were sent to the Department of Pathology for routine histological diagnosis, while (most of the time) the other part after SEM evaluation was also available for post-scanning LM histology using the method of Ayres et al [67]. Tissue processing for SEM was similar to the procedure for cells. Using SEM the natural superficial tissue surface was studied, while consecutive artificial tissue planes were obtained for the study of cell surfaces hidden in the tissue bulk. This was done by a simple

Fig 4
a. SEM of exfoliated normal superficial ectocervical cell illustrating 'basal' cell side architecture found in the normal squamous epithelium of the human cervix and also, the regional variation in surface structure, which is related to contacts with different adjoining cells (see text) (Bar = 1 µm)
b. The 'basal' cell side i.e., the cell side which was orginally directed to the basement membrane, shows globular microvilli and surface grooves, arrow, (Bar = 1µm).

Fig 5
a. An intermediate squamous cell observed in low-magnification SEM (Bar = 1 µm)
b. The high-magnification view reveals microridges and globular microvilli on microridge-like surface elevations, delimited by a terminal bar (Bar = 1 µm).

Fig 6
a. A SEM observation on a folded exfoliated normal ectocervical cell, allowing both sides of the cell to be studied (Bar = 1 µm)
b. At higher magnification the surface difference between the 'basal' cell side and the 'luminal' cell side of the cell is evident, as the cell shows microvilli (and linear grooves) on one side and microridges on the other (Bar = 1 µm).

Fig 7
a. Dysplastic cell with still flat, polygonal cell shape but with obvious nuclear enlargement (Bar = 1 µm).
b. Higher magnification of cell surface microvilli in the area of the atypical, large and globular nucleus. (Bar = 1 µm)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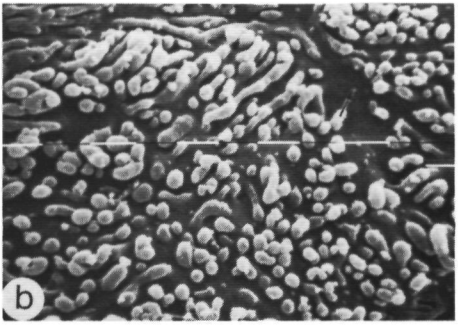
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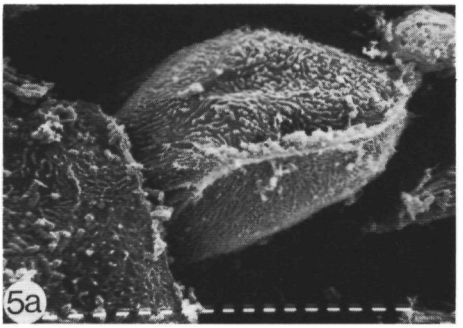
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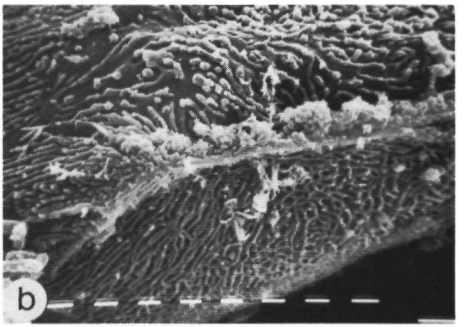
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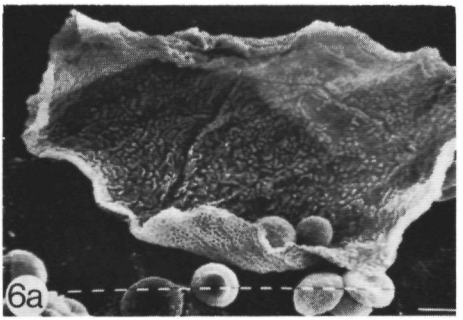
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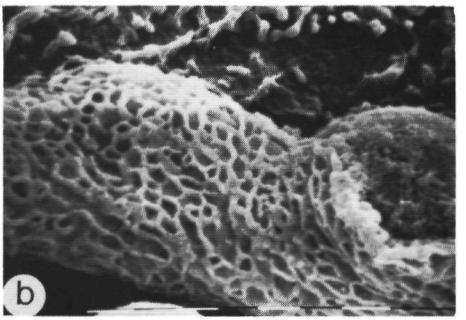
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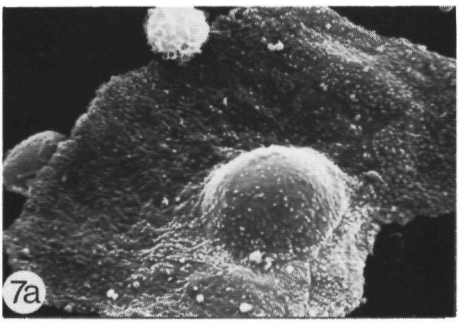
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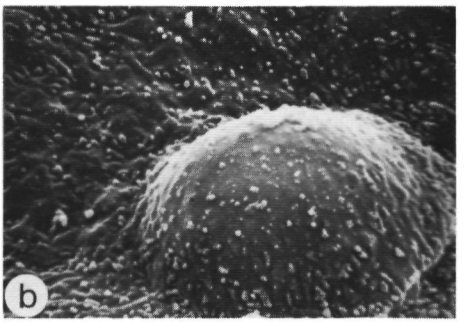
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mechanical tearing technique in the Critical Point (CP) dried phase, and later by a more refined stripping technique, which allowed the study of "complementary" cell surfaces of originally adjacent cells initially located within the tissue. After pressing double adhesive tape against the tissue surface of biopsies, which were fixed and routinely processed for SEM, a layer of epithelial cells is stripped off. Both the sheet of cells on the tape and the remaining biopsy are sputter-coated again, and studied in the SEM. Thus the basal side of a particular cell can be compared with the luminal side of the initally underlying neighbour cell.

(Some details of the methods used as well as some preliminary results have been reported elsewhere [53, 68].)

Results and Discussion

All techniques used gave good preservation of cell surface ultrastructure. Classification of Pap stained cells in LM was possible, although staining was not as optimal as in the routine Pap-stain. Consecutive identification in SEM of LM classified cells was not difficult to accomplish

Normal Ectocervical Epithelium

Normal cervical squamous epithelium shows an orderly tissue organization with a superficial layer consisting of large (30-50 µm in diameter), flat polygonal cells. These superficial cells overlap one another, while many cells are in the process of exfoliation, and therefore, the superficial cells do not form a real continous layer. Cells may exfoliate singly or in clusters The latter are most probably cells still interconnected by desmosomes [69].

The cell surface micromorphology of the free luminal side of normal, mature superficial cells show, characteristically, three distinct features (fig 1-3) viz.,

1. microridges (as described before, e.g., [70])
2. terminal bars, the interconnected and elevated cell boundaries of two laterally adjacent cells (as described before, e.g., [70])
3. linear bars (not described as such before, as far as we know) These cell surface elevations are morphologically similar to the terminal bars, but not situated at the cell border, but on the cell surface. The bars may join each other (see fig. 3). Most probably these linear bars are present at those locations on the cell surface where two (or more) initially more superficially positioned adjacent cells (which have been desquamated already) had their cell borders joining each other Light microscopically these linear bars give the well known dark lines within the cytoplasma, which were sometimes held to be "artefacts or cell-wrinkles".

The surface micromorphology of the non-luminal cell side, the side originally directed to the basement membrane (i.e , the so-called "basal cell side") of normal, mature superficial and intermediate ectocervical cell shows characteristics, not reported before for human squamous cells (see [70]), although in some animal studies (cervix [71], bladder [60]) similar patterns were mentioned, viz ,

1 globular microvilli
2 so-called linear grooves, which are most probably "complementary" to the protruding terminal bars on the luminal cell sides of the subadjacent cells (fig. 4).

Intermediate cervical cells (fig. 5) show essentially a surface ultrastructure similar to that of the superficial cells. However, the microridge pattern and the shape of microvilli can vary to a large extent depending on the degree of maturity of the cells. Here again microvilli do not imply abnormality

The hypothesis that there is such a basal cell-side / luminal cell-side difference in (cervical) squamous stratified epithelium is based on the following observations.

a. LM/SEM parallel studies on sedimented cells revealed that LM normal cell populations showed, under SEM, an almost equal incidence of cell surfaces with microvilli and of surfaces with microridges.
b. LM/SEM consecutively on the same cell showed globular microvilli on LM normal cells from LM normal populations. Moreover, folded - LM normal-exfoliated cells, allowing both cell sides of the same cell to be studied, frequently revealed the cell side difference with SEM (see fig. 6).
c. SEM study of originally neighbouring cell surfaces within the tissue revealed, after intercellularly cleaving with the stripping technique, differing but rather complementary cell surfaces on the two sides of the cleavage plane, viz., microvilli-like structures on one side and microridge patterns on the other.

Squamous metaplasia

The very interesting process of squamous metaplasia is a physiological process in which, starting at the tips of the endocervical columnar villi, the endocervical columnar cells bearing microvilli appear to be converted to large flat new squamous epithelial cells with a microridge *surface pattern on their luminal cell sides.* Immature metaplastic cells show several variations of the microvillous patterns but these abundant microvilli do not imply any abnormality. As cells within this transformation zone may show different degrees of maturation, many closely located cells differ as to cell shape, cell volume and cell surface microvillous pattern.

Cervical Intraepithelial Neoplasia (CIN)

CIN, encompassing the entire group of precursors to invasive epidermoid carcinoma of the uterine cervix, is regarded as forming a fluent continuum of progressively more severe abnormalities. Cell shape, which can still be polygonal and flat (but with obvious nuclear enlargement) in some types of dysplasia (fig. 7) is, like in metaplasia (fig. 8), globular and pleomorphic in the smaller CIS cells (fig. 9). The characteristic luminal cell surface organization of normal superficial cells, which is generally considered, to represent an endstage of squamous cell differentiation or maturation, is progressively impaired. from an increasingly stunted

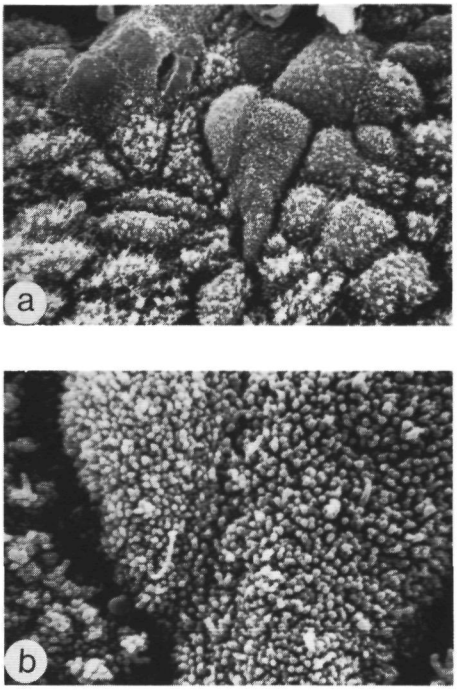

Fig. 8:
a. Tissue organization in early squamous metaplasia. The larger metaplastic cells are situated between smaller cells which still show the endocervical, glandular cell type surface structure. (Bar = 1 µm).
b. The larger metaplastic cells have a closely packed microvillous surface pattern. (Bar = 1 µm).

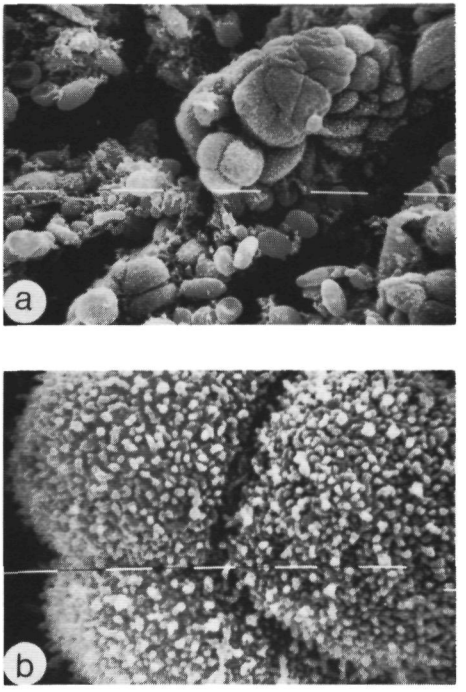

Fig. 9:
a. Low power SEM of carcinoma in situ of the cervix reveals areas with disorganization of the superficial layer which consists of rounded cells of irregular shape and size.(Bar = 1 µm).
b. Cell surface ultrastructure of the free, luminal side of the dome-shaped CIS-cells, showing abundant microvilli and altered cell junctions. (Bar = 1 µm).

microvillous pattern in dysplasia, with intermediate forms consisting of surfaces with both microridges and microvilli, to the well-known tightly packed long microvilli on the bulging surfaces of CIS-cells (fig. 9). Linear bars and terminal bars disappear, intercellular junctions (fig. 10) become ill-defined in CIS, while sometimes in dysplasia lateral cell side specializations in the form of a particular microridge system (fig. 11) on still rather flat cells, are noted. We have the impression that the basal side of the cells also show modification of their cell surface pattern in comparison with normal mature squamous cells. The microvilli look more stunted in dysplasia (fig. 7), and more abundant in CIS.

Thus, the precancerous lesions show a continuous spectrum of modifications. The description we have given is not at great variance with that of other researchers [36, 47-52, 61, 62, 70]. However, the awareness of a "basal"/"luminal" cell side difference will lead to a different interpretation of cell surfaces described, especially when studying exfoliated cells.

In our opinion most studies lack rigid definitions based on a statistical, quantitative formanalytic approach of the notions involved (e.g., microridges, microvilli, long, short, numerous). We are now applying a quantitative method to the analysis of cell surface structures, using a computer aided measuring technique. Until then it will be difficult to tell the difference between e.g. microvilli on CIS cells (fig. 9b) and those on early immature metaplastic cells (fig. 8b). The former have been claimed to be longer and more numerous [70]. Distinguishing CIS and metaplasia is much easier when tissue organization characteristics, pleomorphism of cells and intercellular junction are taken into account.

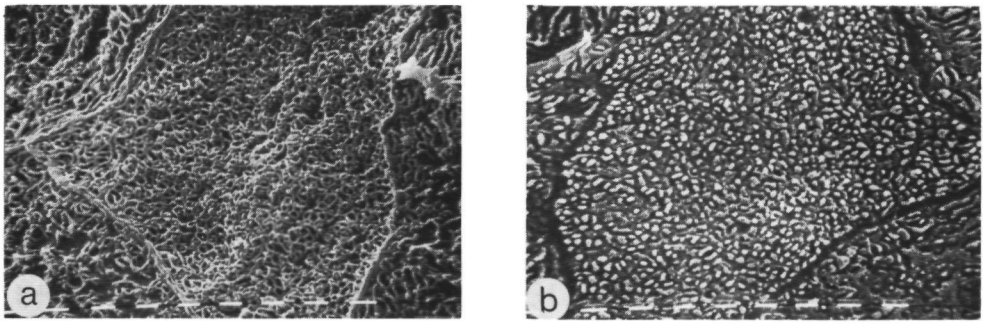

Fig. 10:
Adjoining cell surfaces of normal ectocervical cells within the tissue are observable in SEM after intercellular cleavage of the stratified tissue using a special stripping technique (see text). (Bar = 1 µm). Cells which have been adjacent show 'complementary' surfaces after stripping (compare a with b). Cell surfaces which were directed to the original lumen of the tractus genitalis ('luminal' cell surfaces) situated on the remaining biopsy (a) show a microridge pattern (and protruding bars), arrow, while the cell surfaces which were originally directed to the basement membrane ('basal' cell surfaces) situated on the tape (b), show microvilli (with grooves, arrow, which correspond to the bars).

Regarding the central question of this review the following points, encountered in our study, are of interest.

1. Polar and Regional Differences in cell surface ultrastructure

In addition to surface differences found between the (basal and luminal) sides of the same cell ("cell polarity differences"), there are also surface variations within the region of one cell side. ("regional differences"). This difference is most distinct on the "basal" side of normal and early dysplastic superficial and intermediate cells (fig. 4, 12), where the differing areas are outlined by the "linear grooves". This leads us to the assumption that these differing surface regions have initially been covered by different adjoining cells, causing these regional differences. If so, then care should again be taken to pay attention to the fact that cell surface ultrastructures can never be regarded as the exclusive expression of intrinsic (genetic) cell properties, but always result from the unique interactions of the cell with its neighbours, and other (natural) surroundings. The influence of neighbouring cells on cell topography was elegantly demonstrated in mixed cell aggregates [72].

2. Microridges

Microridges are found on the luminal sides of normal superficial squamous cells, and also on mature (late) metaplastic cells; they constitute an interlacing cell surface system, with several degrees of complexity which relate to the degree of maturation. However, these cytoplasmic ridges were also described on invasive (well- and moderately differentiated) neoplastic superficial squamous cells, whereas in poorly differentiated carcinoma a microvillous pattern is observed [70]. Others [51] have reported dominating patterns of "bizarre" microvilli with or without fragmented microridges. We found many cancer cells to have a rather smooth surface. Cancer cells may thus show microridge-like structures depending on the actual differentiation present in the tumor. Therefore, "microridges" as such (without more detailed description) cannot be considered to imply normality.

3. Microvilli

Where cancer cells cannot be defined as cells totally lacking differentiation (and thus lacking the ultrastructural characteristics of differentiation), the statement that progressive severity of CIN can be characterized as an increasing impairment of cell and tissue differentiation seems to hold true. Also at the SEM level progressive lack of microridges and progressive increase in number of microvilli is found. However, when taking only the surface micromorphology into account the CIS cells would compare well with the early metaplastic cells, even better with the immature (para)-basal cells of the original epithelium. Then the process of carcinogenesis could be described as an impaired conversion of columnar cells to squamous cells, rather than an impaired maturation of the immature squamous cells. This would fit the proposal of Coppleson and Reid [73], that cervical cancer is initiated during the process of metaplasia. The awareness of LM cytologists that there is more then one type of dysplasia, and moreover that there are 3 principal types of CIS as percursor lesions of the three principal types of invasive carcinoma [74], has not been taken into account by many SEM researchers. They may have been studying three processes of oncogenesis, rather than one.

"Microvilli" as such (without more detailed

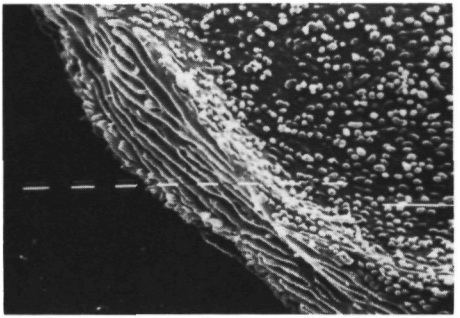

Fig. 11:
Regional variation in cell surface ultrastructure on a dysplastic exfoliated ectocervical cell. Basal cell side pattern with globular microvilli and particular microridge-like surface specialisations on the lateral cell side (Bar = 1 µm).

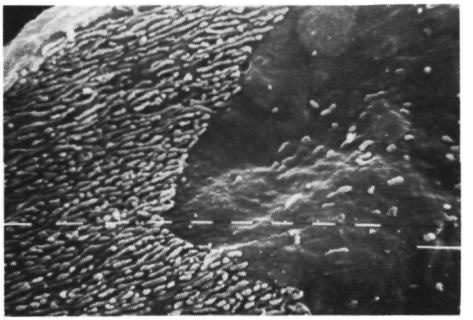

Fig. 12:
Cell contact dependent regional variation in cell surface ultrastructure of a dysplastic cell. (Bar = 1 µm).

description) cannot be considered to be indicative of abnormality, as these cell surface structures are also found on non-malignant cells, viz., normal immature cells (para)basal cells, metaplastic cells) and on normal more superficial squamous cells, on their "basal" side. Microvilli on neoplastic cells have been considered not to be tumor specific, but merely a consequence of rapid epithelial turnover in the neoplastic epithelium which bring immature, deeper lying cells too quickly to the surface [43, 58]. Are long and pleomorphic microvilli then pathognomic of the tumor process, as suggested before [23, 33, 60]? Rubio [49] described the anisovillosis as the most dominant feature of malignant cells, while others [70] describe the CIS cells as having long, numerous closely packed microvilli of variable size and configuration on their surface. Data obtained in large populations producing statistically significant evidence have not been published so far. However, long numerous pleomorphic microvilli exclusively covering the entire cell surface (as described in free floating metastatic carcinoma cells [26-30]) have only been found on CIS cells, and are not pathognomic for cells in invasive cervical cancer.

4. Irreversible Neoplastic Transformation

As CIN lesions (the so-called precursors of cervical cancer) have been shown to persist for years without progression to invasion, (and even regress), most probably many CIN lesions will never become invasive, even when tolerated for many years. Therefore, the most interesting question is: "Do SEM-cell surface characteristics identify that contingent of cells within the total CIN-population, that will irrevocably become invasive? Or in other words can SEM really discriminate those cells that have been irreversible transformed, that have passed the "point of no return", and will progress to cancer. If so, then a prediction about an individual lesion would be possible, which is impossible with LM. SEM would become of paramount importance for optimal patient management.

Based on a review of the relevant literature, and on our own work reported here, the answer has to be negative regarding this question. As this question concerning the oncogenic and metaplastic potential of human (pre-)malignant cells has hardly been posed explicitly until now, much more directed research is needed.

Concluding Remarks

1. Cell surface topography is a dynamic concept. It is not solely an expression of genetic cell properties but it is also related to several other factors and circumstances (e.g., preparation and culture techniques, hormonal stimuli, infections, immuno-factors, neighbouring cells, cell cycle, cell polarity and cell maturation).
2. Cancer cells constitute a very heterogenous group. Carcinoma cells differ from sarcoma cells. Cancer cells in vitro lose many original characteristics. Premalignant cells, superficial cells of invasive tumor, invasive tumor cells at the invasion front, and metastatic cells have different cell (surface) properties.
3. Glandular type carcinomas differ in SEM appearance from squamous type carcinomas. Squamous cell carcinoma may be the result of several different carcinogenetic routes, even cancers of a single anatomical substrate (e.g., the uterine cervix) may originate from different cell types.
4. The points mentioned above make the assessment of SEM studies of cancer cells difficult.
5. Squamous epithelium is the ideal type for the study of early events in oncogenesis. SEM recognizes morphological differences between normal, premalignant and malignant squamous lesions. As LM also establishes these

differences, SEM techniques do not have clear advantages. Until now, SEM (as LM) does not allow predictions to be made about individual lesions, nor does it contribute to staging problems and to the development of a selective therapy.

6. Malignant cells show modifications in their cell surface organization, which theoretically may be relevant to cancer diagnosis, prognosis and therapy. The altered cell surface organization is reflected in detectable changes in cell surface properties, which may be structural (e.g., surface topography; junctional complexes; membrane - associated cytoskeletal components), functional (e g., receptor sites for lectins; anionic charge sites) or immunological (loss of antigens, new, tumor specific antigens) in nature. Several of these surface properties can be studied with SEM techniques (e.g., using surface labeling techniques). It is probably not the most promising approach to the problem, when out of all these cell surface parameters the surface topography is chosen as the object of study in the search for an early marker of irreversible neoplastic transformation and other biomedical applications.

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Discussion with Reviewers

G.D. Wilbanks: Is not the basal-luminal cell side surface difference described by the authors, a tongue-and-groove phenomenon? That is, if one looks at these two opposing surfaces, one has grooves of openings, which probably coincides with the protrusions of microvilli on the corresponding adjacent cell.

P. Veselý: Did you try to improve the insight into morphology of the complementary sides using stereo pairs?

Authors. Yes, we did make stereo pairs, and agree with both reviewers. Comparison of the visual image obtained by studying stereo pairs of the luminal cell side with that obtained by studying stereo pairs of the corresponding basal side of the formerly adjacent, overlying cell, highly improved our insight in the overall spatial organization of the inface between these cells. (Results, i.e., pairs of stereo pairs illustrating this basic fundamental morphological plan of the stratified squamous epithelium in general will be published elsewhere). Essentially, the microridges and bars on the luminal cell side interlock tightly, respectively with the globular microvilli and grooves present on the basal side of the neighbouring cell. In the present paper we mentioned this structural plan only in passing, as we tried to stress the fact that microvilli,

(although globular in shape) can be found on normal squamous epithelial cells, and therefore do not necessarily imply (pre-)malignancy.

B.M. Heatfield: What can be said about the structural and functional status of globular or stubby plasma membrane projections seen on basalar surfaces of ectocervical epithelium, and other epithelia? Are they comparable to microvilli on luminal surfaces of epithelia?
Authors: Globular or stubby plasma membrane projections found on the basalar surface of mature squamous epithelial cells differ in shape from the well known more slender cylindrical extensions found on the free, luminal surfaces of absorptive, secretory or sensory epithelial cells, and also from the microvilli present on dividing cells in vitro, where they may act as membrane storage sites during mitosis. At the moment one can only speculate about the functional status of the globular microvilli that we described. According to the principle of 'optimal design' the spatial organization would give a perfect integrity of the tissue, preventing discruption under shearing stress.

G.D. Wilbanks: It would be very interesting to study the adjacent ectocervical cells from the transmission electron microscope, to identify the location of desmosomes and other types of junctional complexes. Has this been done by the authors or by any other researchers?
Authors: Transmission electron microscopical (TEM) studies of the ectocervical epithelium (text references 52, 64, 65, 69, and also 75-78) report that the cells of the basal and parabasal zone have many microvillous processes, which show desmosomes at their top. The number of desmosomes and gap junctions in the basal, parabasal and intermediate zone is large. Junctional complexes are hardly present or defective in the superficial zone, where the plasma membranes show a fine undulating pattern, and neighbouring cells are interdigitating in a zip-fastener fashion. The TEM studies did not discriminate between microvilli and microridges.

P. Veselý: To which depth (in terms of cell layers) did you observe the polarity of cell sides (basal versus luminal)?
Authors: Study of the surface pattern differentiation of the epithelial cells of the ectocervix revealed that only flattened cells show polarity of cell surface (see also the paper of Davina et al., this volume). Some 7 to 12 pseudo-layers of flattened cells are present in the normal epithelium of the human ectocervix.

Reviewer VI: The phenomenon of the "linear bars" on the surface of cervical cells (of which the authors state, in parentheses, "not described as such before, as far as we know") has been reported before in cervical epithelium in human subjects. (Murphy, Allen, Jordan and Williams: British Journal of Obstetrics and Gynecology 1975, 82: 44-51, cf.p.46 right column).
Authors: The reviewer is right. Bars and grooves (also called crests and furrows, respectively) have been considered erroneously to represent cell borders by many authors (text references 49, 50, and also 79-85), while others 86-88 have interpreted these structures in the right way. However, while the latter authors discriminated between marginally situated 'terminal' bars and more centrally situated 'linear' bars, they did not recognize that bars are present on the luminal side, and grooves on the basal cell side.

L.G. Koss: The appearance of pleomorphic microvilli probably has no prognostic significance.
B.M. Heatfield: Some authors have proposed that pleomorphic microvilli are morphologic markers of irreversible neoplastic transformation in animal and human bladder urothelium. What is your view on this correlation?
Authors: Tumour cells of the primary site are in some instances devoid of microvilli (text reference 28, 54). Thus, there can be epithelial neoplastic transformation without the sign of pleomorphic microvilli, and this is also described in the bladder (text reference 38). On the other hand, pleomorphic microvilli are a surface characteristic of human metastatic epithelial cells present in effusions, and also of many primary (glandular,and undifferentiated squamous cell) carcinomas (text reference 27, 31-53). What remains is the question, whether or not the presence of pleomorphic microvilli on a cell invariably, unequivocally, and early indicates that the process of neoplastic transformation has become irreversible. Until now, this problem remains unsettled. Even Jacobs and co-workers (text reference 37), who (based on their experimental animal model) advocated the clinical use of SEM for early detection of bladder neoplasia, considered the possibility that pleomorphic microvilli "as more cases are examined might possibly be found on the surface of non-neoplastic lesions".

G.D. Wilbanks: Are the alternate cell surface changes as described for normal and dysplastic cervical cells also found in more advanced cells, such as carcinoma-in-situ, or invasive cancer cells?
Authors: No, in our material more advanced cells obtain a more globular shape, losing in this process their cell surface polarity.

G.D. Wilbanks: The authors comment regarding the correlation of the cell surface with the various histologic type of CIN, i.e. those specified by Koss and Reagan, the small cell, large cell type of CIN, but they do not mention it. One questions whether they have made any correlation with such observations and differences of histologic type of CIN.
Authors: This question is the objective of part of our current investigations.

G.D. Wilbanks: It is interesting to note that in the figure of the CIS biopsy, the surface is quite irregular with much cellular debris on the surface. Is this a general characteristic of the more advanced stages of CIN, or is this common in all pre-malignant epithelia from biopsy specimens?
Authors: This phenomenon is encoutered more often in carcinoma-in-situ tissue, and most frequently

in invasive carcinoma, probably due to altered tissue organizations.

G.D. Wilbanks Are the same surface changes found in the exfoliated cells and in the biopsy surface cells of the various lesions?
Authors Yes, when applying "gentle specimen handling"- techniques, as in our study. Cells that have been smeared to the substrate as in normal practice often show cell surface artifacts in SEM. For instance, carcinoma-in-situ cells may even lose their cytoplasm, giving naked nuclei surrounded by debris.

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Chapter 3 Tannic Acid Binding of Cell Surfaces in Normal, Premalignant, and Malignant Squamous Epithelium of the Human Uterine Cervix

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Alterations in tannic acid (TA) binding capacity of cell surface carbohydrates in normal, premalignant, and malignant squamous epithelium of the human uterine cervix have been studied using electron microscopic visualization in combination with microdensitometric evaluation

While in normal epithelium there is distinct binding in four to five cell layers of the deep intermediate zone, cells of carcinoma in situ and invasive cancer lesions lack TA binding In moderate dysplasia an intermediate reacting pattern is found

Deep intermediate cells in areas bordering the carcinoma in situ lesions do not show any binding, although their ultrastructure cannot be distinguished from similar cells in normal tissue

The TA deposition within the deep intermediate zone is probably related to the presence here of glycoprotein containing membrane-coating granules

The finding that TA binding discriminates between cells in normal squamous epithelium and morphologically normal cells in juxtaposition with lesional areas in premalignant and malignant epithelium opens the possibility for a more reliable cytologic diagnosis of cervical epithelial neoplasia

KEY WORDS uterine cervix, epithelium, malignancy, ultrastructure, tannic acid

INTRODUCTION

To demonstrate the presence of premalignant and malignant lesions in the uterine cervix the Papanicolaou staining of cervical smears is at present most commonly used Cervical smears, however, do not always contain sufficient premalignant and/or malignant cells to allow reliable diagnosis On the other hand it has recently been shown that intermediate cells of a cytologically normal appearance possess diagnostic potential [1–4] In order to find additional methods for cytologic diagnosis our studies concentrate on surface characteristics of the squamous epithelial cells In previous studies we were able to demonstrate that the scanning electron microscopic cell surface characteristics are not useful as markers of irreversible neoplastic transformation [5,6] Cytochemical analysis of cell surface alterations may be a more promising approach because the altered cell surface

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properties of malignant cells are thought to be related to altered cell behavior [7]

Many studies have been performed which reveal that cells modulate their cell surface glycoconjugates during malignant transformation (for recent reviews see Bhavanandan and Davidson[8] and Monsigny et al [9]) For almost all glycoconjugate studies biochemical techniques were used Few investigators have traced the structure related localization of the glycoconjugates In particular, little information is available about alteration of cell surface glycoconjugates in malignancy of the uterine cervix [10]

For the light microscopic visualization of glycoconjugates, a number of methods with varying specificity exist [11] However, for the ultrastructural visualization only a few methods are available [12] Tannic acid (TA) has often been used in electron microscopic studies [13–17] It is a suitable stain for the carbohydrate rich cell surface [14]

Biopsies from the transformation zone of the cervix uteri from patients with a premalignant or malignant lesion and from control patients were studied The TA staining pattern of the cells in layers of the stratified squamous epithelium was analyzed Microdensitometric techniques have been used to obtain an objective registration of the staining results Particular attention was paid to the epithelium adjacent to the lesions with an apparently normal appearance

MATERIALS AND METHODS

Specimen Preparation

Biopsies from the cervix uteri of cycling patients with moderate dysplasia (2 patients) carcinoma in situ (5 patients), and squamous cell carcinoma (3 patients) were taken after colposcopic examination The surveyed and sampled areas contained the (pre)malignant lesion As control material, biopsies were taken from 12 patients without cervical abnormalities who had to undergo extirpation of the uterus for other reasons One half of each biopsy was cut into strips with an epithelial covering of about 1 mm^2 These were fixed according to Wagner with minor modifications [14] The fixative containing 2% paraformaldehyde, 2 5% glutaraldehyde, and 3% TA (Merck, DAB 7) in 0 1 *M* sodium cacodylate buffer was used (pH 6 3 940 mOsm, 20°C) After 2 h fixation the solution was changed to the fixative without TA (pH 7 2, 935 mOsm 4°C, 16 h) As a control part of the tissue strips were fixed without exposure to TA The specimens were washed in 0 1 *M* sodium cacodylate buffer and postfixed in 2% OsO_4 in Palade buffer (pH 7 4 20°C, 1 h) Finally, the specimens were rinsed dehydrated in graded ethanol followed by propylene oxide, and embedded in Epon 812 according to standard procedures Diagnostic evaluation was performed comparing the 1 μm toluidine blue stained Epon sections with the hematoxylin eosin-stained paraffin sections of the other half of the biopsy Ultrathin sections of TA treated and control tissue were either left unstained or further contrasted with lead citrate and studied in a Philips EM 301 microscope at 60 kV Standardized microscopic and darkroom conditions were maintained in producing photographs to achieve a linear relationship between electron dose and film optical density [18]

Microdensitometry

For objective determination of the TA staining results, a computer controlled scanning microdensitometer [BioPEPR (Biological Precision Encoding and Pattern Recognition)] was used This allowed determination of the optical density (OD) from the EM negatives of the TA-stained and unstained specimens A detailed description of the BioPEPR system has been given by Zahniser [19] whereas Linders[18] described its use in electron microscopy for mass determination of isolated biological objects In the present study, part of a micrograph (Fig 1*a* and *b*) was imaged on the BioPEPR television screen to enable coarse selection of the area to be measured From this area a binary image was made A horizontal line indicated the location of the measurements The binary image was an aid in the fine positioning of the line for the eventual measurements The binary image of the selected area was photographed in order to document the exact place of the measurements The OD of the structures crossing the line was determined, stored, and subsequently displayed as a line profile (Fig 1*c*) The ratio of the OD value of the cell surface to that of an adjacent intracellular membrane (nuclear

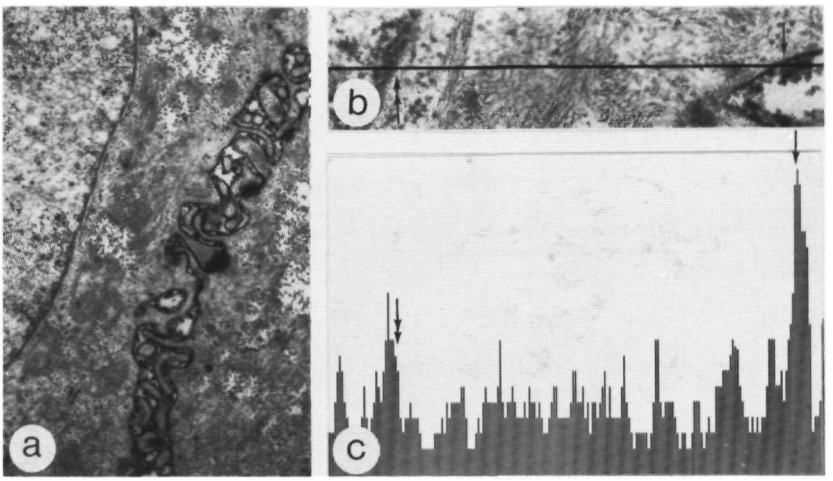

FIG. 1 *Microdensitometry for determining tannic acid (TA) binding of a deep intermediate cell (TA treated). Parts of electron micrograph* (a, b) *were imaged on BioPEPR TV-screen to enable coarse selection of area to be measured. Line in* (b) *indicates location of measurements. Line profile* (c) *shows distribution of optical densities of structures crossing line. Optical density of cell surface* (arrow) *clearly differs from that of nuclear envelope* (double arrow). (a) ×*10,300;* (b) ×*36,000.*

envelope; mitochondrial outer membrane) was used as a measure of the intensity of the TA staining. Only perpendicularly sectioned membrane profiles were studied. For control purposes, the OD was also determined in non-TA-treated cells (Fig. 2).

RESULTS

The evidence for adequate penetration of TA in the specimen was the positive staining of desmosomes, basal lamina, and collagen. From comparison with the TA untreated tissue it appeared that TA did not penetrate the cell cytoplasm, which is in accordance with the results of Wagner.[14] Therefore, the intensity of the staining of the cell surface could be monitored by comparing its electron opacity with that of the intracellular membranes.

Normal Epithelial Cells

In the normal squamous epithelium of cycling women, 20 to 25 cell layers are present, which can be divided into four zones (Fig. 3*a*). The basal zone is formed by one layer of cuboidal to cylindrical cells. The 3- to 5-cell layers thick parabasal zone consists of polyhedral cells. The intermediate zone possesses 4- to 5-cell layers of large, round cells (deep intermediate cells) and 4- to 5-cell layers of more flattened cells (upper intermediate cells). The superficial zone consists of 7- to 12-cell layers of flattened, partially exfoliating cells with pyknotic nuclei.

Tannic acid treatment of normal squamous epithelium reveals distinct differences in the staining pattern of the cell zones. The surface of basal (Fig. 3*b*) cells only shows weak staining. From the parabasal (Fig. 3*c*) to the intermediate zone the TA positivity of the cell surfaces increases. The reaction is most evident in the 4 to 5 deep intermediate cell layers (Fig. 3*d*). The cell surface and intercellular material of these cells is strongly stained. The upper intermediate cells (Fig. 3*e*) and superficial cells (Fig. 3*f*) do not show surface staining. The strongly stained deep intermediate cells possess membrane coating granules (MCG) located near their cell surface (Fig. 4*b*). The MCG diameter is approximately 0.1–0.2 μm; the granules do

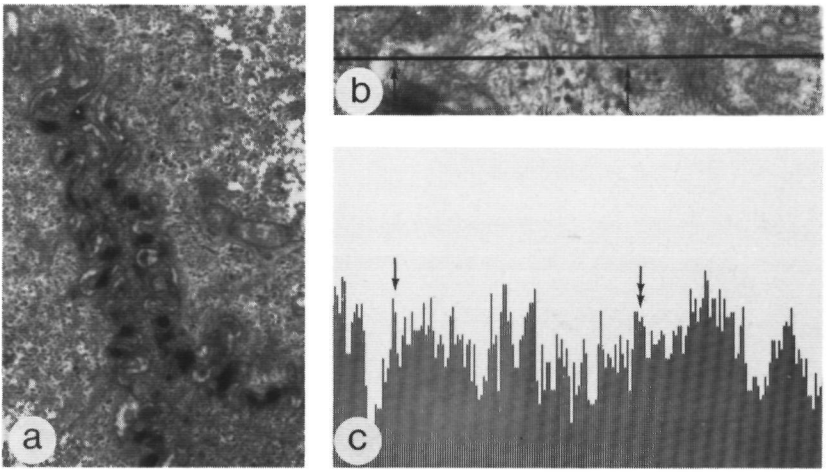

FIG. 2 *Microdensitometry for determining tannic acid binding of deep intermediate cell (non-TA-treated). For explanation of technique see Fig. 1. Cell surface* (arrow) *possesses about equal optical density as mitochondrial membrane* (double arrow). (a) *×10,300;* (b) *×36,000.*

not show distinct internal lamellations. The TA positivity rapidly diminishes in the transition zone from these deep intermediate cells (Fig. 4*b*) to the upper intermediate cells (Fig. 4*c*). In the cytoplasm of the upper intermediate cells no MCG were encountered (Fig. 4*c*).

Premalignant and Malignant Epithelial Cells

The TA staining pattern in premalignant and malignant epithelium deviates from the normal pattern. In moderate dysplasia, cells with a TA-positive cell surface do occur. These cells are only present in small focal groups, which are distributed in the transition area of the parabasal to the intermediate zone (Fig. 5). The stained cells possess less electron-dense cytoplasm than the unstained cells. In the carcinoma in situ lesions, no cells with a TA-positive cell surface have been observed. In addition, intermediate cells adjacent to the carcinoma in situ lesion with a morphologically normal appearance do not show surface TA positivity (Fig. 6). Invasive carcinoma cells possess TA-negative surfaces irrespective of the degree of the differentiation of the squamous cell carcinomas.

Microdensitometric Data

Even when only perpendicularly sectioned membrane profiles were studied, the optical densities (OD) of membrane structures showed small differences, which were most likely due to differences in section thickness. Therefore the ratio of OD of the cell surface (OD_{cs}) to that of an adjacent intracellular membrane (OD_{im}) was used as a measure of the intensity of the TA staining. The ratio was 1 for cells in control tissue (non-TA-treated) including the deep intermediate cells (Fig. 2; $OD_{cs}/OD_{im} = 1.0 \pm 0.1$; $n = 11$).

In TA-treated normal squamous epithelium the basal, parabasal, upper intermediate, and superficial cells show no differences in OD of surface and of intracellular membranes ($OD_{cs}/OD_{im} = 1.0 \pm 0.1$; $n = 14$). However, the OD of deep intermediate cells is significantly higher than the density of the intracellular membranes (Fig. 1; $OD_{cs}/OD_{im} = 1.6 \pm 0.1$; $n = 6$). Carcinoma in situ and squamous carcinoma cells

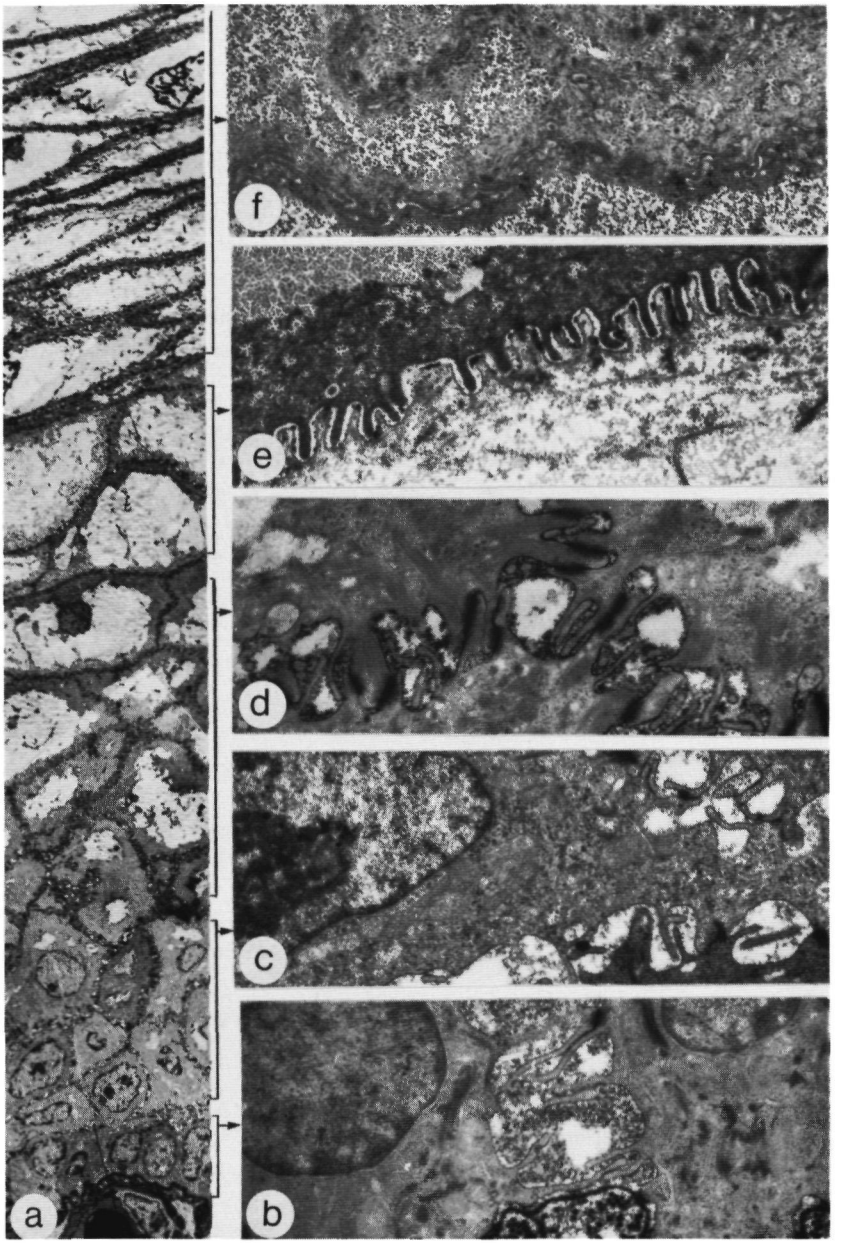

FIG. 3 *Tannic acid cell surface staining in normal squamous epithelium of uterine cervix* (a). *Basal* (b) *and parabasal* (c) *cells show only weak staining; deep intermediate cells* (d) *demonstrate strongest staining; upper intermediate* (e) *and superficial* (f) *cells are not stained.* (a) ×*1200;* (b–f) ×*10,300.*

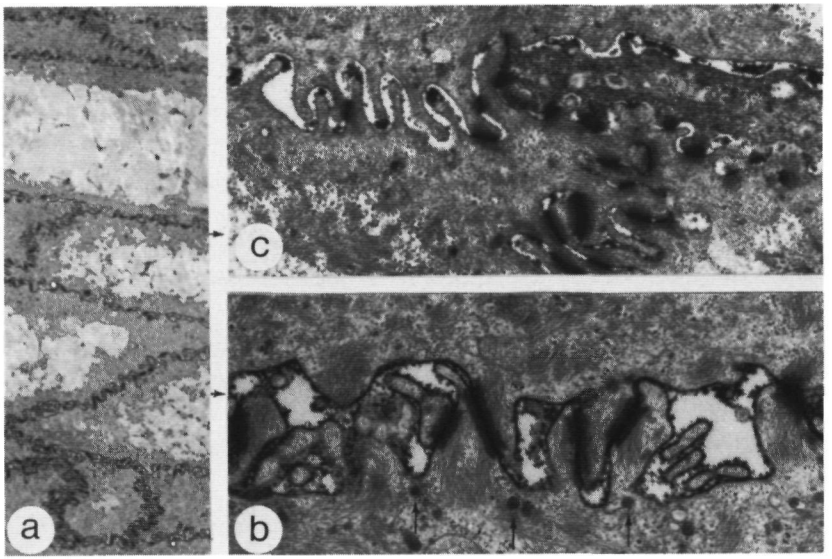

FIG. 4 *Transition area* (a) *from deep intermediate cells* (b) *to upper intermediate cells* (c). *Deep intermediate cells possess tannic acid-stained cell surface and membrane-coating granules* (arrow) *in apical cytoplasm; upper intermediate cells lack these characteristics.* (a) X*3200;* (b, c) X*16,000.*

either treated with TA or not all show a ratio of 1 ($OD_{cs}/OD_{im} = 1.0 \pm 0.1$; n = 10), which means that the cell surface is left unstained after TA treatment.

DISCUSSION

Tannic acid was originally introduced in electron microscopy for the stabilization of tissue components, i.e., prevention of extraction during tissue processing.[13,20] It appeared that it also acts as a mordant between osmicated tissue structures and lead stains.[14,21,22] Mostly TA is considered as a stain for proteins and polypeptides.[13,20,23,24] This has been shown by treating the tissue with trypsin and collagenase.[15] Phosphatidylcholines[17,25] and protein-bound hyaluronic acid[16] also demonstrated a substantial increase in contrast after TA treatment. Thus TA appears to be a stain for several carbohydrates. Moreover, TA reactivity depends on specific conditions of the substances mentioned above, especially the pH.[20,23]

Tannic acid indicates differences in cell surface properties within the normal squamous epithelium of the uterine cervix. The surface and intercellular material of the 4-5 layers deep intermediate cells in all control patients are strongly stained by TA in contrast to the surface of the basal, parabasal, and superficial cells. It is striking that the TA surface reactivity coincides with the occurrence of MCG in the cells. Differences in cell surface staining have also been demonstrated in the stratified squamous epithelium of the normal human esophagus using the periodic acid-silver technique.[26] In the latter study, mucoproteins have been shown to be present in the intercellular space of the upper prickle and lower functional cell layers. Because periodic acid-silver staining was performed using ultrathin sections, the MCG in these cell layers were also stained.[26] In view of the fact that the occurrence of the MCG is restricted to the upper prickle and lower functional layers, it seems justified to conclude that these layers correspond to the deep inter-

mediate cell layers of the ectocervical epithelium. It also seems obvious to relate the surface reactivity of these cell layers to the occurrence of MCG in the epithelium. This is further supported by the fact that TA positivity is missing when the MCG no longer occur in the intermediate cells. Our observations on the occurrence of MCG are in agreement with early ultrastructural observations in the squamous epithelium of the uterine cervix.[27] From cytochemical studies it has been concluded that MCG in nonkeratinizing epithelia contain glycoproteins,[28,29] whereas those in keratinizing epithelia also contain phospholipids.[30,31] The content of MCG is extruded into the intercellular space, where it probably provides the efficient permeability barrier found in most such epithelia.[30,32,33] It is also probable that the MCG and its extruded product are involved in the process of epithelial maturation and regulation of epithelial thickness and desquamation.[34] In conclusion, the location in the epithelium, the rapid diminishment, and the involvement of glycoproteins point to a relation between MCG exocytosis and TA staining in the deep intermediate zone.

Our results show that the cell surface TA-binding properties in premalignant and malig-

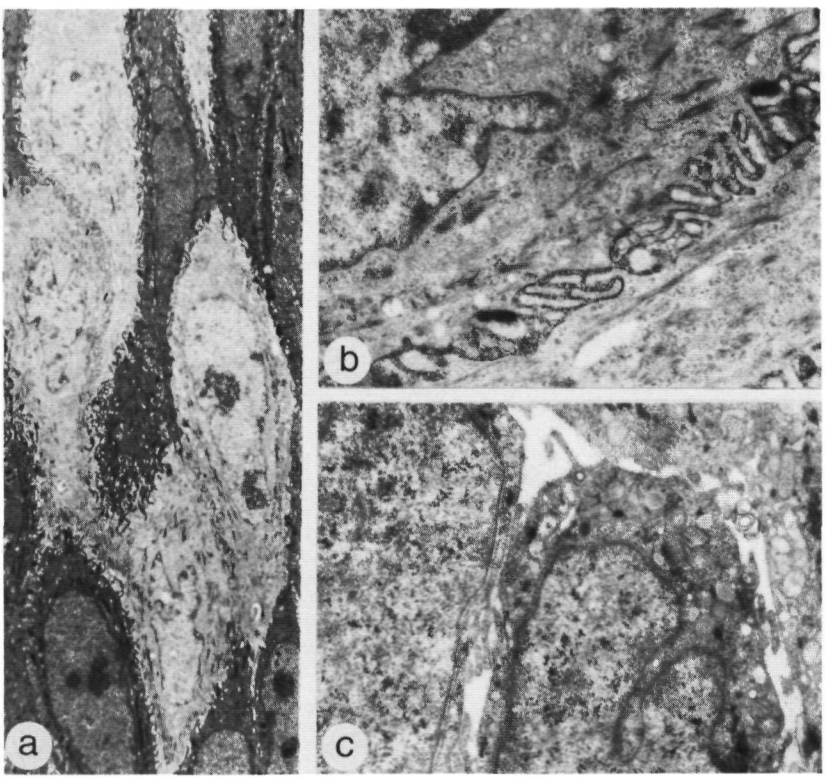

FIG. 5 *Tannic acid binding in moderate dysplasia. Focal groups* (a) *of TA-positive cells* (b) *are present among negative cells* (c) *in transition area from parabasal to intermediate zone. Cell surface staining coincides with occurrence of a less electron-dense cytoplasm,* (a) ×*2400;* (b, c) ×*9200.*

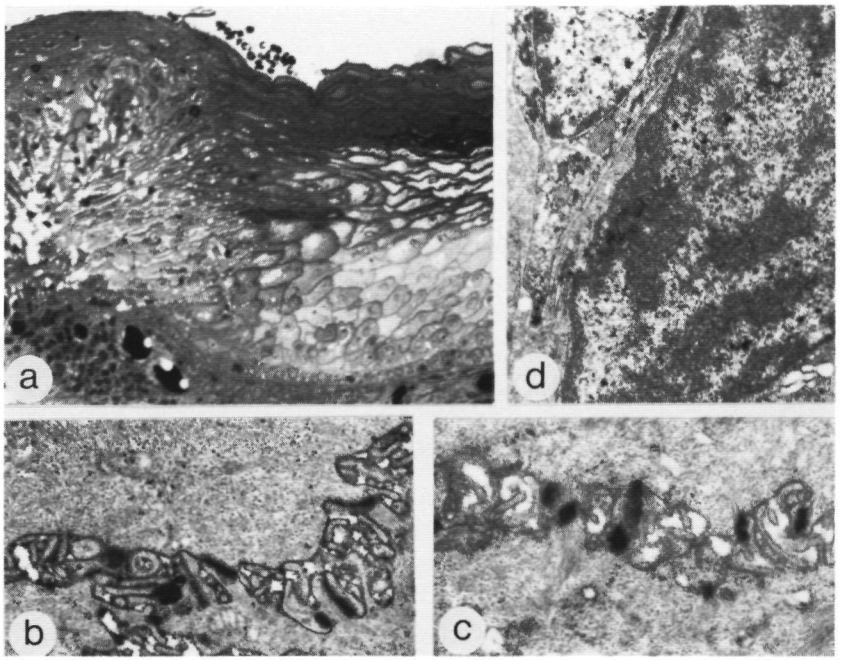

FIG. 6 *Morphologically normal appearing epithelium adjacent to carcinoma in situ lesion* (a). *Deep intermediate cells far from lesion* (b) *possess a TA-stained cell surface, and similar cells adjacent to lesion* (c) *are not stained by tannic acid; premalignant cells* (d) *also remained unstained.* (a) *X80;* (b–d) *X7900.*

nant cervical epithelium are different from those in normal squamous epithelium. While the normal epithelium contains a continuous zone of about five layers of positively reacting cells, epithelia with moderate dysplasia contain only some focal groups of positive cells. The carcinoma in situ lesions and squamous cell carcinoma lesions do not possess positive cells at all. Many studies have shown, as reviewed by Bhavanadan and Davidson[8] and Monsigny et al.,[9] that malignant transformation is associated with alterations in cell surface glycoconjugates. In a number of tumors the amount of cell surface glycoconjugates appeared to alter with neoplasia.[8,9] For almost all these studies, biochemical techniques were used. Only a small number of carcinoma studies concerned structural localization of glycoconjugates. Blood group antigens have been lost from the cell surface in premalignant and malignant lesions of the epithelium of the uterine cervix,[10] of the oral cavity,[35] and of the bladder.[36] In rat bladder epithelium, lectins have been used to localize surface glycoproteins[37]; an increased number of binding sites was found and it was suggested that this alteration occurred prior to morphologic changes in bladder carcinogenesis.[37]

The diagnosis of premalignancy and malignancy of the uterine cervical epithelium is commonly based on the presence of atypical cells in Papanicolaou-stained smears. The many intermediate cells present in the smears, however, are not considered. This study has shown that the surface of morphologically normal deep intermediate cells in areas adjacent to carcinoma in situ lesions is not stained by TA in contrast to similar cells in normal epithelium.

This finding may have diagnostic significance Indeed, evidence exists that points to a diagnostic value of intermediate cells It has been shown that glycogen loss precedes the morphologic expression of malignancy [38] The suggestion was made that the glycogen content of the intermediate and superficial cells in particular can serve as a useful tool in the early diagnosis of recurrence [38] Evidence for the diagnostic value of intermediate cells has also been presented by demonstrating the existence of "computer recognizable subvisual" markers in light microscopically normal intermediate cells in smears from patients with various stages of cervical cancer [1-4] When altered intermediate cells occur earlier and/or in greater number than the premalignant cells, this may have important diagnostic value Moreover, a prognostic significance may be attributed to such a finding when their occurrence is related to the behavior of the lesion (progressive, stable, regressive) It remains to be established whether our findings possess this diagnostic and prognostic value and what the effect is of non-neoplastic conditions More specific methods for this study are more suitable, as for instance the binding of lectins to the cell surface

In conclusion, the present study has revealed alterations in the TA binding capacity of cell surfaces in association with the occurrence of (pre)malignancy The difference in occurrence of cells with TA stained cell surfaces in and near premalignant lesions and in normal epithelium suggests that the cell surface carbohydrates may be useful for a more reliable cytologic diagnosis of cervical neoplasia

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Chapter 4 Concanavalin A-Peroxidase Labelling in Cervical Exfoliative Cytopathology: 1. Labelling of Normal Squamous Cells and the Detection of Cancer

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ABSTRACT

The lectin binding capacity of the cell surface of normal flattened exfoliated epithelial cells of the uterine cervix was investigated looking for differences between specimens from normal and cancer patients. The method used was a *modified concanavalin A-horseradish peroxidase (ConA-HRP)* labelling procedure.

Both normal and cancer specimens contain labelled as well as unlabelled usual flattened cells. There is a distinct difference between the labelling intensity of labelled and that of unlabelled cells. Quantification of the labelling results has been achieved using a light microscope equipped with a computerized video system. Apparently healthy persons, having a percentage of labelled flattened cells between 54% and 94% (mean=73%, s.d.=10%, n=40), were totally discriminated by this method from the cancer patients. These patients with a histologically confirmed squamous cell carcinoma, showed a labelling percentage between 10% and 22% (mean=15%, s.d.=4%, n=10). Hormonal factors, such as phase of cycle and pill use, appeared to have no significant influence.

Statistical analysis revealed that at least 99% of all healthy persons will have a labelling percentage above 45%, while at most 1% of the cancer patients will show a labelling percentage above 30%. When choosing the labelling percentage of 45% as critical value, the ConA-HRP labelling might serve as an

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additional detection method for cancer of the uterine cervix. Moreover, as it is based on the abundantly present normal cells, and not on the often scarce abnormal cells, the method is not liable to sampling and screening errors.

Key words:
uterine cervix, exfoliative cytology, cancer, detection, concanavalin A.

INTRODUCTION

For the early detection of human cervical cancer the screening of Papanicolaou stained cytological smears is a method which is widely used but which is not completely reliable. This is because of the sampling and screening errors that may arise (Bartels et al., 1979), and because of the small number of (pre)malignant cells present in a smear (Bibbo et al., 1975, 1976a, 1976b). The cytological screening method would be considerably improved if the many morphologically normal cells present in the smears, could be used to extract information concerning a possibly ongoing disease process. In this respect interesting results have already been obtained through the analysis of the nuclear-chromatin pattern of apparently normal epithelial cells (Bibbo et al., 1981; Burger et al., 1981, Sincock and Middleton, 1983). The red cell adherence test of leucocytes present in smears (Wagner and Noltenius, 1983) also seems promising. With the same aim, we have directed our attention, in particular, to the cell surface of normal epithelial cells.

In previous studies, it was established that both reversible and irreversible neoplastic alterations are not characterized by specific morphological cell surface alterations even when looking at the fine structural level (Davina et al., 1981, Kenemans et al., 1981). On the other hand, while studying cervical biopsies with cytochemical techniques, it was found that morphologically normal epithelial cells have altered surface properties when they bordered a (pre)malignant lesion. Whereas in normal epithelium, the surface of intermediate cells possesses a strong affinity for tannic acid, intermediate cells bordering a premalignant lesion do not show this feature (Davina et al., 1984).

In the present study, exfoliated epithelial cells have been investigated for their reactivity with a cell surface labelling technique. Use has been made of concanavalin A-horseradish peroxidase (ConA-HRP) as a label. It is known that

the lectin ConA specifically binds to oligosaccharides containing mannoside and glycoside residues (Sharon and Lis, 1972). The conditions for optimal cell surface labelling have been established and the labelling method has been applied to cervical cell suspensions from both healthy individuals and from patients with a squamous cell carcinoma.

MATERIALS AND METHODS

Cytopathological material

Exfoliated cells were collected from the uterine cervix of 40 healthy individuals in which repeated cytological smears had not revealed any epithelial abnormality, and from 10 patients with a histologically confirmed squamous cell carcinoma. The age of the patients varied between 20 and 65 years. Data were collected regarding the menstrual cycle phase and about the possible use of oral contraceptives at the moment of cell collection.

Preparation of cervical cell suspensions

Epithelial cells from the cervical portio were collected with a plastic Ayre spatula. The cells were suspended in 10 ml 0.01 M phosphate buffered saline (PBS), pH 7.4, containing 0.15 M NaCl and 2.5 mM KCl. Proper disaggregation of the cells was achieved by the syringeing technique described by Oud et al. (1984a). Part of the cells to be stained for screening according to Papanicolaou, were deposited onto microscope slides as described by Oud et al. (1984b). The greater part of the cells was pelletized by centrifugation at 400 g for 5 minutes and subsequently resuspended in 5 ml of a 0.1% glutaraldehyde fixative in 0.1 M phosphate buffer, pH 7.4. Fixation was for 10 minutes at room temperature. After repeated centrifugation, the cells were resuspended in 5 ml 0.1 M NH_4Cl in PBS for at least 10 minutes in order to block remaining aldehyde groups which might cause non-specific labelling (Brown and Revel, 1976). Then, the cells were rinsed twice by alternate pelletizing (400 g, 5 minutes) and resuspension in 5 ml PBS. The number of cells per milliliter, determined with a Coulter Counter^R, appeared to vary from 50,000 to 3,000,000. It was found that the cell suspensions could be stored in PBS at 4°C for up to 10 days without noticeable effect on the labelling results.

Labelling procedure

The ConA-HRP labelling procedure as described by Bernhard and Avrameas (1971) was modified as follows. In order to find optimal concentrations for ConA

(grade III, Sigma^R) and HRP (grade II, Sigma^R), cells were treated with varying concentrations of ConA (10, 100, 500, 1000 µg/ml) in PBS at 37°C for 2 hours, followed by incubation with HRP (5, 10, 25, 100 µg/ml) in PBS at 20°C for 45 minutes. The optimal concentration for ConA was found to be 100 µg/ml and for HRP 10 µg/ml. After incubation in ConA followed by the HRP-treatment, cells were stained at room temperature for 10 minutes with a solution containing 0.05% 3,3-diaminobenzidine-hydrochloride (DAB, Sigma^R) and 0.01% H_2O_2 in Tris-HCl buffer, pH 7.6 (Graham and Karnovsky, 1966). After each step, the cells were washed with PBS (2x10 minutes). For control purposes either the incubation in ConA alone or the incubations in ConA and HRP were omitted. An additional control was performed by adding 0.2 M alpha-D-methylmannoside (Sigma^R) to the solutions of ConA and HRP (Bernhard and Avrameas, 1971).

After labelling, the cells were deposited onto microscope slides as indicated above, counterstained with haematoxylin, dehydrated in ethanol, mounted in DePeX (Gurr^R), and examined with a light microscope at a magnification of 10x25x. It was found that the labelling intensity was not noticeably influenced by the cell density of the suspension.

Electron microscopy

In order to establish the cellular distribution of the ConA label, part of the ConA-HRP-DAB treated cells were prepared for transmission electron microscopy. These cells were pelletized and resuspended in 2% low gelling temperature agar (LTD agar, Miles^R) at 40°C and pelletized again. After coagulation of the agar, the embedded pellet was cut into blocks of about 1 mm^3. These were treated with 2% osmium tetroxide in Palade buffer, pH 7.4, at room temperature, for 1 hour. Finally, the specimens were rinsed, dehydrated and embedded in Epon 812 according to standard procedures. Unstained ultrathin sections were studied in a Philips EM 301 microscope at 60 kV.

Assessment of labelling results

After ConA-HRP-DAB treatment all squamous cells were found to be stained to some extent. HRP-DAB treated control cells showed a staining, the intensity of which was always distinctly less than the intensity of labelled cells. Henceforth, cells were defined labelled when their staining intensity was stronger than the staining intensity of the HRP-DAB treated control cells.

Labelled and unlabelled cells were distinguished using the Rapid Optical

Scanning System University Nijmegen (ROSSUN). This is an integrated image recognition system used for the rapid automated screening of flat surfaces (microscopical slides, films) for the presence of recognizable patterns with a diameter larger than 1 μm. The system consists of a light microscope (Leitz Orthoplan) equipped with a Hamamatsu C1000 video system, a DEC-PDP11/23 minicomputer and required interfaces. For our studies the Hamamatsu system was used in the sliced mode (analogous/digital resolution 1 bit). The grey-scale was set at the level at which all cells of the control specimen were not depicted, in contrast to the ConA-HRP-DAB labelled cells which were depicted in the sliced mode at the screen. The total number of cells was counted after switching to the normal setting of the video system (8 bit).

Quantification of the labelling results was performed by establishing the relative number of labelled flattened cells. Pathological cells and other epithelial cells were not involved. Only free separately lying cells were taken into account, i.e., cells that were (almost) not overlapping other epithelial cells. Double counting was prevented by screening the slides according to a fixed meandering pattern. For establishment of the labelling results, three different series of 100 cells on each slide were screened. The labelling percentage of a patient is the mean of the 3 countings. Using this method the accuracy of the labelling percentage was estimated to be 1.3% (Davina et al., 1985). In a few cases only 100 or 200 cells per slide were available for counting.

Statistical analysis

Student's two-samples test was used to examine the difference between the labelling percentages of healthy individuals and those of the cancer patients. For the control group a lower bound (one-sided guaranteed coverage lower tolerance limit) for the labelling percentage has been established, below which are probably at most 1% of the healthy individuals. The chosen level of confidence was 90% and a normal distribution was supposed. In an analogous way an upper bound has been established for the group of cancer patients.

The possible effect of the phase of the menstrual cycle on the labelling results, was examined by analysing the difference between the labelling percentage of healthy individuals in the follicular phase (day 6-10) and of healthy individuals in the luteal phase (day 14-30)with the use of Student's

test for two samples. In addition, a one-way analysis of variance was applied with respect to the labelling percentages on different days during one cycle of one healthy individual. The Student's test was also used to examine the difference between the percentage of pill using healthy individuals and non-pill using healthy individuals.

RESULTS

The screening of Papanicolaou stained specimens revealed that the cell suspensions almost exclusively consisted of superficial and intermediate cells. As a consequence of the disaggregation technique, only a small number of the flattened epithelial cells occurred in sheets. In the specimens of the healthy individuals occasionally some metaplastic cells and cylindrical cells were present, whereas basal and parabasal cells were only incidentally encountered. The specimens from the cancer patients also contained premalignant and malignant cells.

Healthy individuals

Cells were defined labelled when their staining intensity was stronger than the staining intensity of all HRP-DAB treated control cells. Although the ConA-HRP-DAB staining of cells varied from weak to strong, there was a clear difference between labelled and unlabelled cells. The distinct difference in labelling of the flattened cells is illustrated in figure 1. From our observations, we gained the impression that superficial cells seemed to be labelled less frequently and their labelling intensity was less if compared with the

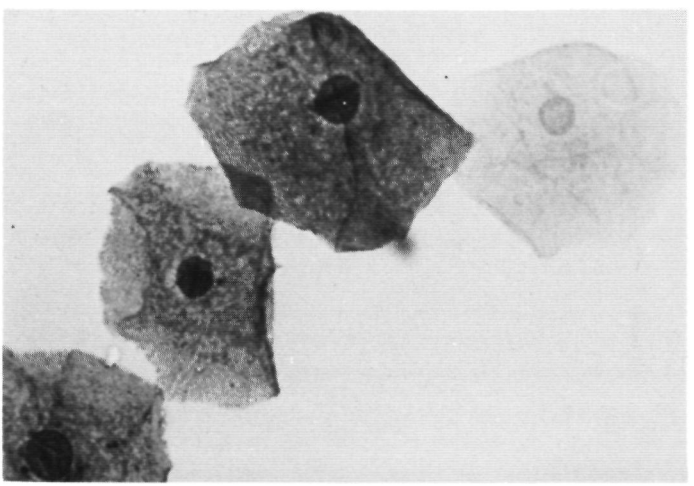

Figure 1.
Light micrograph of ectocervical squamous epithelial cells after labelling with ConA-HRP-DAB. A clear difference is shown between labelled and unlabelled cells (magn. 1000x).

intermediate cells, but this was not quantitatively evaluated. Electron microscopical study revealed that labelled cells had a thick layer of homogeneous electron-dense precipitate all along the cell surface (figure 2). Prolonged ConA-treatment of cells occasionally resulted in penetration of the lectin into the cell, as is demonstrated by the presence of some precipitate in the perinuclear space. All mature metaplastic cells showed a more intense labelling than did the cylindrical and immature metaplastic cells.

The percentage of labelled flattened cells in the 40 healthy individuals appeared to vary from 54 to 94, with a mean of 73 and a standard deviation of 10 (figure 3). When a normal distribution was supposed and a level of confidence of 90% was chosen, the one-sided guaranteed coverage lower tolerance limit with respect to the 1%-point of the distribution of the labelling percentage was 45%. This means that under equal conditions probably at most 1% of all healthy individuals will show a labelling percentage below 45%.

Cancer patients

In the cell suspensions of patients with a squamous cell carcinoma all cylindrical, metaplastic, premalignant and malignant cells were labelled. The labelling intensity of the carcinoma in situ cells and carcinoma cells was stronger than the labelling intensity of the dysplastic cells. Only a small part of the flattened cells was found to be stained stronger than the HRP-DAB treated control cells. Here too, the superficial cells seemed to be labelled

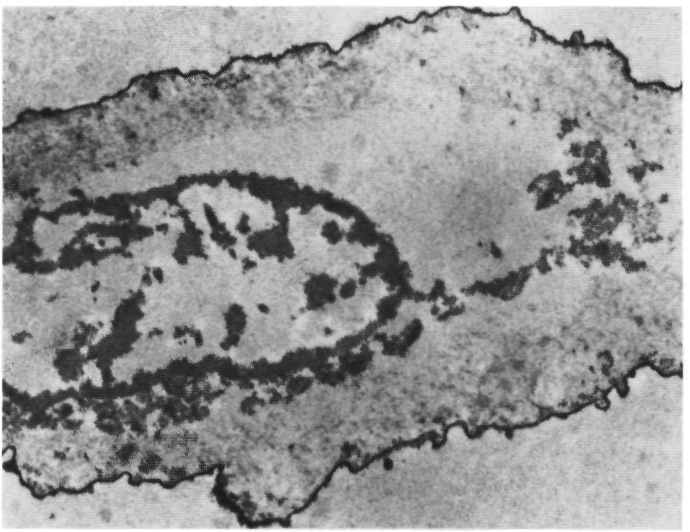

Figure 2.
Electron micrograph of an ectocervical squamous epithelial cell labelled after ConA-HRP-DAB treatment. The cell shows a thick layer of homogeneous electron-dense precipitate all along the cell surface (magn. 10,000x).

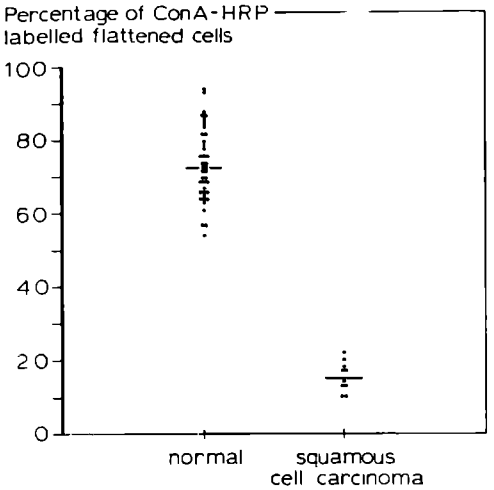

Figure 3.
Distribution of percentages of ConA-HRP labelled flattened cells from healthy individuals and from squamous cell carcinoma patients; the horizontal bar indicates the mean of the group.

less frequently than were the intermediate cells.

The percentage of the labelled flattened cells of 10 squamous cell carcinoma patients appeared to vary from 10 to 22, with the mean of 15, and a standard deviation of 4 (figure 3). This labelling percentage significantly differs from the mean labelling percentage of healthy individuals according to the two-samples test of Student ($p < 0.001$) (table 1). A one-sided guaranteed coverage upper tolerance limit with respect to the 99%-point of the distribution of the labelling percentage has also been determined. Supposing a normal distribution and using a level of confidence of 90%, this limit was 30%. This means that under equal conditions probably at most 1% of all cancer patients will show a labelling percentage above 30%.

The labelling percentages of the healthy individuals and of the cancer patients are given in figure 3. As can be inferred from this figure, the ConA-HRP labelling method discriminates completely between the group of healthy individuals and the group of cancer patients under consideration.

Table 1.
Mean percentages of ConA-HRP labelled flattened cells from healthy individuals and from patients with a squamous cell carcinoma of the uterine cervix.

histological diagnosis	number of women	mean (±s.d.)
normal	40	73 (±10)
squamous cell carcinoma	10	15 (± 4)

Table 2.
Labelling percentages on different days during one menstrual cycle of one healthy woman.

day of cycle	6	7	8	11	13	14	15	20
labelling percentage	77	78	76	73	68	71	77	69

Influence of hormonal state

An attempt has been made to trace any eventual effect of the hormonal state on the labelling percentage. Table 2 shows the labelling percentages at different days during one cycle of one healthy individual. According to a one-way analysis of variance by making use of 3 separate countings of 100 cells per day, the hypothesis of equal labelling percentages for different days had to be rejected ($p=0.003$). However, no clear relation with the phase of the menstrual cycle could be established.

Moreover, when the various healthy individuals using no oral contraceptives were considered, no significant difference could be established between the specimens from the follicular phase (day 6-10) and the luteal phase (day 14-30) by Student's two-sample test ($p=0.47$) (table 3). However, it is noted that again a lower labelling percentage is observed for the luteal phase.

The labelling percentages of healthy individuals using oral contraceptives and of postmenopausal women did not deviate much from the percentages of cycling women (table 3). According to Student's two-sample test, no significant difference could be found between healthy pill using women and healthy non-pill using women ($p=0.25$). For the latter test each healthy individual known as a non-pill user, was considered ($n=28$, mean=72%, standard deviation=10%).

Table 3.
Mean percentages of ConA-HRP labelled flattened cells from healthy individuals with a different hormonal state.

hormonal state	number of women	mean (±s.d.)
follicular phase (day 6-10)	7	75 (±10)
luteal phase (day 14-30)	13	71 (±10)
oral contraceptives	7	77 (± 9)
postmenopause	3	71 (± 5)

DISCUSSION

The present study has revealed that cervical cell suspensions from healthy individuals as well as from patients with a cervical squamous cell carcinoma contain a fairly constant fraction of ConA-HRP labelled flattened epithelial cells with a morphologically normal appearance. The difference between labelled and unlabelled cells is very distinct although the labelling intensity may vary. Although the variation in labelling percentage within each group of patients is rather large, a significant difference has been found between the percentage of labelled flattened cells in the control group and the percentage in the cancer group. This implicates that the ConA-HRP cell surface labelling of the normal flattened epithelial cells completely discriminates between the healthy individuals and the cancer patients under consideration.

Several questions arise from this study such as the reasons for labelling of only part of the flattened cells, the reasons for the existence of a clear difference between labelled and unlabelled flattened cells, and the reasons for the decrease of the labelling percentage in the case of malignancy. Answering these questions was not the main purpose of this study. They merit further investigation. The main purpose of this study has been to find an useful method in detection of cancer of the uterine cervix that has less sampling and screening errors than the Papanicolaou method.

Part of the flattened epithelial cells cannot be labelled with the ConA-HRP labelling method. In addition, it is our impression that superficial cells are labelled less frequently than are the intermediate cells. Possibly, these observations may be explained by an external irritation of the epithelium by injurious agents in the cervico-vaginal lumen, which results in the alteration or loss of oligosaccharide residues which are responsible for binding of the lectin ConA. The permeability barrier in the epithelium may determine the depth of penetration of the potentially injurious agents. It is known that beneath the most superficial cell layers of the stratified squamous epithelium there is an effective barrier for selective exclusion of luminal macromolecules presumably provided by cellular structures as secreting membrane coating granules (Hill et al., 1982), tight junctions (Elias et al., 1977), and interdigitating cell surface structures (Davina et al., 1981). This intercellular permeability barrier was found to be a sharply demarcated zone in the squamous epithelium (Hill et al., 1982), which may explain the

existence of a clear difference between labelled and unlabelled flattened cells. Variation in the number of cell layers lying above the permeability barrier, may be responsible for the variation in labelling percentage within each group of patients, even within one patient.

The ConA-HRP labelling method did not show clearly variation related to the phase in the menstrual cycle, to the use of oral contraceptives, and to the postmenopause. Nevertheless to some extent, a relation between the labelling percentage and the cycle phase may exist. Such a relation is difficult to show, because in general only one measurement per women was available. In addition, the level of the labelling percentage around which the variation appears, differs between individual patients.

A number of studies has shown that in various tissues alterations in cell surface glycoconjugates may occur during malignant transformation (for reviews see Bhavanandan and Davidson, 1982; Smets, 1982). These alterations are also supposed to have a diagnostic value for various epithelial tissues like the uterine cervical epithelium (Davidsohn et al., 1973; Bonfiglio and Feinberg, 1976), the epidermis (Graem, 1982; Hyun et al., 1984), the oral epithelium (Dabelsteen et al., 1983), the urothelium (Young et al., 1979; Yokoyama, 1980; Sadoughi et al., 1980; Richie et al., 1980), the breast epithelium (Voyles et al., 1978; Newman et al., 1983; Howard et al., 1981), and the pancreas epithelium (Raedler et al., 1983). Almost all these studies showed a decrease of labelling of the malignant cells in comparison with the normal epithelial cells. This is in contrast with our observations of intensely labelled carcinoma cells. The above mentioned studies have also not revealed alterations of the morphologically normal cells of the cancer patients. Our study has demonstrated that the number of apparently normal cells with a detectable amount of ConA binding sites is decreased when a malignant lesions is present in the epithelium.

The explanation of the decrease of the labelling percentage in case of malignancy is not obvious. It cannot be due to radio- or chemotherapy treatment, because the cancer patients were not receiving such treatment. It is possible than an increased external irritation in the cervico-vaginal lumen is responsible for a shift of the permeability barrier to the basal lamina causing the decrease of labelled cells. Moreover, the number of labelled cells may decrease as a result of the direct influence from the malignant lesion to

the normal cells adjacent to the lesion. Various studies have shown the possibility of a malignant lesion influencing the enzymatic action or the proliferative activity of the adjacent normal epithelium causing a redistribution of cell surface glycoconjugates or a hydrolysis and addition of oligosaccharide residues (for reviews see Bhavanandan and Davidson, 1982, Smets, 1982). In this respect it is interesting to note the relationship between acid phosphase activity in the cervical squamous epithelium and the release of cell adherence. Ductu et al. (1980) have found that acid phosphatase activity was mainly present in the superficial part of the normal squamous epithelium and in malignant cells of the uterine cervix. A general function has been proposed that involves acid phosphatase activity for sublethal modification of cell periphery and hence a release of cell adherence thus enabling cells to partly release themselves from surrounding structures and permit increased mobility (Sjogren, 1984). In cervical tumor cells, Marshall et al. (1978) found elevated levels of some of the enzymes involved in glucose metabolism, this being presumably the result of an increased metabolism in these cells. This observation is consistent with data of Das and Roy-Chowdhury (1981). They have shown that the glycogen metabolism of normal epithelial cells also was altered in the presence of a (pre)malignant lesion. In a previous study, we have demonstrated that cell surfaces of deep intermediate cells in areas adjacent to carcinoma in situ lesions, have lost their affinity for tannic acid in contrast to similar cells in normal epithelium (Davina et al., 1984). Bamford et al. (1983) showed positive staining for epithelial membrane antigen in cervical neoplastic lesions, and in the adjacent normal squamous epithelium, whereas normal epithelium away from the lesion and normal epithelium in healthy individuals was negative. It is known that even the leucocytes present in the cervical epithelium with a (pre)malignant lesion possess altered surface properties (Wagner and Noltenius, 1983). In other studies, it was shown that part of the morphologically normal intermediate cells possess a deviant pattern in their nuclear chromatin when a premalignant or malignant lesion is present (Bibbo et al., 1981; Burger et al., 1981; Sincock and Middleton, 1983). All these studies point to a possible "environmental" effect of a lesion on the "normal" cells adjacent to the lesion.

The ConA-HRP labelling method of cervical cells as described in this paper may offer a good supplementary test for the detection of cervical cancer because an abundant number of cells for detection is available, and because the

(pre)malignant cells, the number of which is not always adequate, are not necessary for this test. The ConA-HRP labelling seems to allow a clear distinction to be made between healthy individuals and cancer patients in an objective, rapid and easy way. Using a labelling percentage between the guaranteed lower bound for the 1%-point of the distribution for healthy individuals (45%) and the guaranteed upper bound for the 99%-point of the distribution for cancer patients (30%) as a discriminative level, the specificity and the sensitivity of the ConA-HRP labelling method both amount at least 99%. It is concluded that the ConA-HRP labelling of normal epithelial cells may serve as an additional detection method to the Papanicolaou method for cancer of the uterine cervix. It is interesting to examine the percentage of ConA-HRP labelled flattened cells in premalignancy, and to assess the usefulness of the labelling method in the detection of cervical cancer. This is currently under investigation.

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Chapter 5 Concanavalin A-Peroxidase Labelling in Cervical Exfoliative Cytopathology: 2. Routine assessment of labelling results

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ABSTRACT

Concanavalin A-peroxidase (ConA-HRP) labelling of exfoliated cells of the uterine cervix have been shown to possess clinical significance in the detection of cancer. In the present study, a more simple method is used instead of the earlier applied complicated method. These two procedures for the assessment of the labelling results are compared.

The first method is an objective machine-aided assessment procedure, consisting of a light microscope connected with a video system used in the sliced mode. The second is a more subjective method using human visual assessment with a light microscope only. The latter method would be suitable for routine use, if it shows similar ConA-HRP labelling results as obtained with the machine-aided procedure.

In comparison with the machine-aided assessment procedure, the visual assessment procedure is less accurate. Moreover, the visual assessment is accompanied by intraobserver (between-day) and interobserver variations. Although the discriminatory capacity in the detection of cancer patients is significantly lower for the human visual assessment procedure, this difference is small. It is of clinical relevance that in general a complete discrimination of healthy individuals and cancer patients is still possible. Therefore, visual assessment with a light microscope only, is preferred because of its simple equipment, which allows this procedure to be used as a routine method.

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Key words:
uterine cervix, exfoliative cytology, cancer, detection, concanavalin A.

INTRODUCTION

For the detection of cancer of the uterine cervix concanavalin A-horseradish peroxidase (ConA-HRP) labelling of normal squamous epithelial cells has recently been introduced by us (Davina et al., 1985). This cell surface labelling procedure has been proposed as an addition to the standard method in cervical exfoliative cytopathology, viz., the Papanicolaou method. The introduced method is based on the existing difference in ConA-HRP labelling of apparently normal flattened cells from healthy individuals and cancer patients. In this initial study, the labelling results were established using an objective, machine-aided assessment procedure (Davina et al., 1985).

For routine use on a wide scale, a simple visual assessment procedure seems more suitable. However, it is known that all human visual assessment procedures of alterations in cytological and histological patterns, are liable to considerable interobserver and intraobserver differences (Bacus et al., 1982, Castleman and White, 1980). For this reason there is a general tendency to introduce computer-based image analysis systems (Sherman et al., 1984; Zahnizer et al., 1979). However, the diagnostic procedure proposed by us does not imply the recognition of varying patterns of neoplasia. Such patterns usually show a continuous transition from a less to a more atypical character. The ConA-HRP method, on the other hand, requires a simple yes-no decision, i.e., a cell is either labelled or not labelled. In the machine-aided procedure this decision could be made using the analogous/digital converter of the ROSSUN in a one bit sliced mode (Davina et al., 1985). If labelling results could be established in a reliable way using a normal light microscope only, the ConA-HRP method could advantageously be applied as a routine method in any cytopathological laboratory.

The present study investigates whether a direct human visual assessment procedure has a similarly high discriminatory capacity in the detection of cancer patients as has been earlier established for the machine-aided assessment procedure (Davina et al., 1985). To this end, the accuracy of both procedures has been determined as derived from the field variation. The field variation appears when several fields with 100 cells in one specimen are

counted. Moreover, it has been studied in how far the assessment of the labelling results depends on counting on different days (intraobserver variation), and on counting by different observers (interobserver variation).

MATERIALS AND METHODS

Cytopathological material

Cells collected from the portio of the cervix were suspended in a buffer solution. Part of the cell population was stained according to Papanicolaou for independent classification by a cytopathologist. Positive cytology was always confirmed by histology. The remaining cells were successively incubated with solutions of ConA, HRP, and diaminobenzidine (DAB) for labelling of cell surface glycoproteins. Full details of the ConA-HRP labelling procedure and the preparation of permanent slides have been described previously (Davina et al., 1985).

For comparison of the machine-aided and the visual assessment procedures, the same slides were used as described earlier (Davina et al., 1985), i.e., specimens from 40 apparently healthy individuals, and from 10 patients with a squamous cell carcinoma. For the determination of the intraobserver variation cell suspensions from three healthy individuals, from patients having either dysplasia (n=4), carcinoma in situ (n=4), squamous cell carcinoma (n=1), or who were earlier treated in a conservative way for dysplasia or carcinoma in situ (n=4), were used. For the determination of the interobserver variation three of the abovementioned specimens were used, viz. a specimen from a healthy individual, from a patient with a carcinoma in situ, and from a treated patient.

Assessment of labelling results

MACHINE-AIDED ASSESSMENT Labelled and unlabelled cells were distinguished using the Rapid Optical Scanning System University Nijmegen (ROSSUN). This is an integrated image recognition system used for the rapid automated screening of flat surfaces (microscopical slides, films) for the presence of recognizable patterns with a diameter larger than 1 μm. The system consists of a light microscope (Leitz Orthoplan) equipped with a Hamamatsu C1000 video system, a DEC-PDP11/23 minicomputer, and required interfaces. For our studies the Hamamatsu system was used in the sliced mode (analogous/digital resolution: 1 bit). The grey-scale was set at the level at which all cells of

the control specimen were not depicted, in contrast to the ConA-HRP-DAB labelled cells which were depicted in the sliced mode at the screen. The total number of cells was counted after switching to the normal setting of the video system (8 bit).

Quantification of the labelling results was by determination of the relative number of labelled flattened epithelial cells. Overlapping cells and non-flattened cells including pathological cells were not considered. Double counting was prevented by screening the slides according to a fixed meandering pattern. For establishment of the labelling result, three different series of 100 cells on each slide were screened and the average labelling percentage was calculated. Due to variation in cell density of the specimen, the screened areas (fields) varied. In a few cases only 100 or 200 cells per slide were available for counting.

HUMAN VISUAL ASSESSMENT Labelling results were obtained by screening with a light microscope only, using the same slides as assessed with the machine-aided procedure. In order to discriminate between labelled and unlabelled cells, the staining intensity of the cytochemical control cells (HRP-DAB treated) was taken as criterion unlabelled. Concerning the measurements of the group of healthy women and the cancer group for comparison of both methods, only one observer was involved.

Statistical analysis

Differences in labelling percentages as determined by the machine-aided and visual assessment procedures were examined. The Student's paired sample test was used separately for the control group and the cancer group.

The FIELD VARIATION for both procedures was studied. This is the variation which appears when a number of fields (100 cells) in one specimen is screened (same day, same observer). The field variation has to be known for the determination of the optimal number of cells to be counted in a specimen. This variation was defined as the standard deviation of the field countings and estimated by pooling the results from different specimens. The field variation of both procedures was compared with each other using the signed rank test of Wilcoxon.

The ACCURACY of both procedures was expressed as the standard error of the

mean (s.e.m.) of the field countings for a specimen and can be derived from the field variation (for 3 field countings s.e.m. = field variation/$\sqrt{3}$).

The existence of an INTRAOBSERVER VARIATION in the labelling percentage as related to visual assessment on different days by the same observer, was examined. This variation which is the variation after eliminating the field variation, was defined as the standard deviation of the theoretical day means. Field countings of each of 16 specimens were carried out by the same observer on 5 different days. A two-way analysis of variance according to a mixed model (with specimen as a fixed factor, and day as a random factor) was performed where the results from the separate fields were used as scores. Also a joint estimate, based on the two-way layout, for the intraobserver variation has been presented.

The INTEROBSERVER VARIATION in the labelling percentage as related to visual assessment by different observers was studied in a similar way. The interobserver variation was defined as the standard deviation of the theoretical observer means. Field countings of each of 3 specimens were done by 10 persons. Each observer counted the specimens on the same day. Again two-way analysis of variance according to a mixed model (with specimen as a fixed factor and observer as a random factor) was performed.

RESULTS

Comparison of mean labelling results

The mean of the labelling percentages of healthy individuals as determined by one observer using the visual assessment procedure and measuring on different days, was found to be 2.4% lower than the earlier determined percentage using the machine-aided assessment procedure (table 1). These percentages differ significantly. For cancer patients, the mean of the labelling percentages using the visual assessment procedure was 0.5% higher than the percentage determined with the machine-aided assessment procedure (table 1). However, this difference was not significant.

Field variation

Data about field variation as encountered for the healthy individuals and for the cancer patients as well as for the combined groups, are presented in table 2. Although the field variation in the machine-aided assessment is about 1%

Table 1.
Comparison of the two methods for assessment of the percentages of ConA-HRP labelled flattened cells, use is made of healthy individuals and patients with a squamous cell carcinoma, p-values indicate the results of Student's paired sample test.

histological diagnosis	number of women	mean labelling percentage (±sd)		p-value
		machine-aided	visual	
healthy	40	73 (±10)	71 (±11)	0.001
squamous cell carcinoma	10	15 (± 4)	16 (± 5)	0.64

lower than in case of the visual assessment, the difference is significant for the separate groups as well as for the combined group. The question arises whether the field variation is related to the height of the labelling percentage. The F-test of variance-ratio shows that a significantly higher variation for the control group exists compared to the cancer group when the visual assessment procedure is used ($p=0.03$), but that this is not the case when the machine-aided assessment procedure is used ($p=0.18$).

Accuracy

The accuracy of the determination of the labelling percentage of a given specimen is expressed as the standard error of the mean of three countings of 100 cells. Disregarding the effect of a possible difference in field variation between different levels of labelling percentages, the accuracy has been calculated on the basis of the combined group of the control and cancer patients. The accuracy of the machine-aided assessment procedure is 1.3%, and the accuracy of the visual assessment procedure is 2.1%.

Table 2.
Field variation. Comparison of the field variation of the two methods for assessment of the percentage of ConA-HRP labelled flattened cells of healthy individuals and patients with a squamous cell carcinoma; p-values indicate the results of the signed rank test of Wilcoxon.

histological diagnosis	number of women	field variation		p-value
		machine-aided	visual	
healthy	40	2.3	3.8	<0.001
squamous cell carcinoma	10	1.9	2.6	0.04
healthy + sq. cell carc.	50	2.3	3.6	<0.001

Table 3.
Intraobserver variation. Variation in labelling as a consequence of counting of 16 specimens by the same observer at 5 different days, using the visual assessment procedure.

specimen number	1	2	3	4	5	6	7	8
labelling percentage (lab. perc.)	87	82	75	72	72	69	64	62
field variation of lab. perc.	2.5	3.6	2.8	4.2	2.7	3.9	5.2	4.1
intraobserver variation of lab. perc.	0.5	1.4	4.6	3.1	2.4	1.8	3.9	6.2

specimen number	9	10	11	12	13	14	15	16
labelling percentage (lab. perc.)	61	55	42	33	28	26	25	10
field variation of lab. perc.	3.3	3.0	2.6	3.2	4.5	3.4	2.9	2.5
intraobserver variation of lab. perc.	3.2	2.5	1.4	5.6	0.0	0.8	2.3	0.0

Intraobserver variation

The variation in the determination of the labelling percentage as a consequence of counting specimens by the same observer on different days using the visual assessment procedure, is shown in table 3. The separate results of the 16 specimens analysed, are ranged from a high to a low mean labelling percentage. The specimens include samples from healthy individuals, and from patients with dysplasia and carcinoma lesions. These data do not show clearly that the field variation and the intraobserver variation is related to the height of the labelling percentage. The field variation based on the combined data of the 16 specimens is estimated to be 3.5%. According to a joint two-way analysis of variance averaged over all 16 specimens, the intraobserver variation is found to be significant ($p=0.02$). It also has appeared that a

Table 4.
Interobserver variation. Variation in labelling as a consequence of counting of 3 specimens by 10 different observers, each at the same day, using the visual assessment procedure, the specimen numbers refer to the numbers mentioned in table 3.

specimen number	2	6	14
mean labelling percentage of 10 observers	83	63	30
field variation of labelling percentage	4.7	6.2	3.3
interobserver variation of labelling percentage	5.8	9.2	4.9

difference between two fixed days can depend on the specimen (interaction test, $p < 0.001$). As suggested, for each specimen the same variance of the theoretical means per day for the labelling percentages can be assumed. If next the covariance between the means per day for two specimens is assumed to be the same for each set of two specimens, the intraobserver variation based on the combined results is estimated to be 3.0%.

Interobserver variation

Table 4 presents the separate results from the 3 specimens which were used for the establishment of the interobserver variation. It is not shown clearly that the field variation and the interobserver variation are related to the height of the labelling percentage. Besides, the field variation is supposed to be the same for the different observers. The field variation based on the combined results is estimated to be 4.9%. A joint two-way analysis of variance shows that averaged over the 3 specimens, the labelling percentage established by the different observers varies significantly ($p < 0.001$). Also the interaction test gives a significant result ($p<0.001$). Assuming an analogously symmetric covariance matrix for the theoretical means per observer of the labelling percentages as done for the intraobserver variation with respect to the day means, the interobserver variation is estimated to be 6.9%.

DISCUSSION

Assessment of the percentage of ConA-HRP labelled flattened epithelial cells using a light microscope only (human visual assessment), is accompanied by the introduction of extra variations as compared to the machine-aided assessment. The machine-aided assessment of the labelling results offers a distinct discrimination between labelled and unlabelled cells. The simple yes-no decision (labelled-unlabelled) is possible using the video system in the sliced mode, and setting the grey-scale at the level at which all cells of the HRP-DAB treated control specimen are not depicted. The machine-aided assessment does not consider the varying labelling intensity of the labelled cells. On the other hand in the visual assessment, the observer should evaluate the HRP-DAB treated control cells before counting the ConA-HRP-DAB labelled cells. When cells with varying labelling intensity are observed, it is possible that the observer does not exactly know the background staining of the control cells. This results in extra variations, which have been shown in the present study. However, it is of clinical relevance that the complete

discrimination between healthy individuals and cancer patients is still well possible using the visual assessment procedure. In the following, the extra variations of the visual assessment procedure will be discussed in relation to its applicability for the detection of cancer patients.

The present results show three different joint estimates for the field variation of the visual assessment procedure. On the basis of measurements from all healthy individuals and cancer patients, this field variation amounts to 3.6%, whereas the intraobserver analysis yields a value of 3.5%, and the interobserver analysis a value of 4.9%. It is noteworthy that the first two estimates, which are nearly equal, orginate from the same experienced observer, while the latter estimate originates from a number of mostly less experienced observers. This might indicate that the field variation depends on the observer to some extent.

The total variation for the visual assessment procedure as a consequence of counting a limited number of fields and counting at different days, can be calculated. For the one observer, involved in the intraobserver analysis and the comparison of the controls and cancer patients, this total variation amounts to 3.6%. If however, the counting of each specimen has been carried out on the basis of only 100 cells instead of 300 cells, the total variation would be 4.6%. On the contrary, counting of 9 yields of 100 cells per specimen would yield a total variation of 3.2%. So, taking into account the extra effort needed for counting more cells, the labelling percentage of a specimen based on counting of 3 fields has been chosen.

Although the intraobserver and the interobserver analyses did not show clearly decreasing variations with decreasing labelling percentages, the cancer patients gave rise to a significantly lower mean field variation relative to the controls. If the intraobserver variation reduced proportionaly at the same rate, the total variation with respect to the one observer (counting 300 cells per specimen) could be estimated as 2.6% for the cancer patients and as 3.9% for the controls. This means that the variation of the measuring result generally is not related to the height of the labelling percentage very much.

With regard to the interobserver variation, it may be expected that this includes intraobserver variations. In fact the interobserver variation (6.9%) was found to be considerably larger than the intraobserver variation (3.0%).

As expected, the human visual assessment is liable to extra variations, and therefore it discriminates less between the control and the cancer groups. This has also been observed in the diagnosis of urothelial cells by Sherman et al. (1984). Their study concerns the difficult recognition of patterns of neoplasia and shows a continuous transition from a less to a more atypical character. But the present study concerns the discrimination between labelled and unlabelled cells which in principal is a simple yes-no decision. Although the visual assessment possesses a significant lower discriminatory capacity in the detection of cancer patients than the machine-aided method, for the one observer under consideration the visual assessment procedure also allows the complete discrimination with regard to the healthy individuals and the cancer patients. It is of importance that the size of the total variation in the visual assessment is small in relation to the wide interval between the two groups. False-positive and false-negative observations still do not occur. Therefore, the visual assessment procedure is preferred because its simple equipment, which allows this procedure to be used as a routine method. We may expect that the ConA-HRP labelling as a method for detection of cancer of the uterine cervix in addition to the Papanicolaou method, can be performed by most laboratory technicians without special training in microscopy. The well-trained cytotechnologist can concentrate on a more careful microscopic screening of the cases with a low percentage of ConA-HRP labelled flattened cells.

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Chapter 6 Feasibility of a Concanavalin A-Peroxidase Labelling Method to Detect Cancerous and Precancerous Lesions of the Uterine Cervix

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ABSTRACT

For the detection of cancerous and precancerous lesions in cervical cytopathology, the feasibility of a concanavalin A-peroxidase (ConA-HRP) labelling procedure was tested and compared with the Papanicolaou method. To this end, the percentage of labelled flattened epithelial cells with a morphologically normal appearance present in cervical cell suspensions, was determined.

It was found that the mean labelling percentage of the control group was 71% (s.d.=11%). The means for mild, moderate, and severe dysplasia groups were respectively 54% (s.d.=19%), 48% (s.d.=13%), and 44% (s.d.=16%). The mean for the carcinoma in situ group was 32% (s.d.=11%), and for the squamous cell carcinoma group 16% (s.d.=5%). It appeared that the labelling percentage gradually decreases with increasing atypia of the epithelium as confirmed by histologically observation. A complete discrimination was found between healthy individuals and cancer patients.

In a follow-up study it was found that the mean labelling percentage did not alter in cases of an unchanged stage of disease. A re-establishment of the normal ConA-HRP labelling percentage often appeared once the cancerous or precancerous lesion was treated.

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In conclusion, the ConA-HRP labelling method can be considered as a supplementary method to the Papanicolaou method for the early detection of cervical cancer. It reduces the effect of sampling and screening errors of the Papanicolaou method, and it allows a more objective cytological diagnosis. In addition, the method may possess prognostic significance.

Key words:
uterine cervix, exfoliative cytology, cancer, detection, concanavalin A.

INTRODUCTION

Cytological screening for cervical cancer and its precursor lesions using Papanicolaou stained smears, is an approved and generally applied method. Nevertheless, results obtained with this screening method are not completely reliable because of the well documented occurrence of sampling and/or screening errors (Bartels et al., 1979).

We recently reported about the application of another promising approach in screening for cancer of the uterine cervix (Davina et al., 1984; Davina et al., 1985a). This method is based on the detection of cytochemical alterations of the cell surface of normal flattened squamous epithelial cells. The method uses the fact that cells contain considerable amounts of oligosaccharide residues on their surface which specifically bind to lectins such as concanavalin A (Davina et al., 1985a). The number of cells with a considerable amount of these binding sites appears to differ significantly between healthy individuals and squamous cell carcinoma patients. A high percentage of labelled flattened epithelial cells has been shown to be present in the former group and a low percentage in the latter. Statistical analysis revealed that at least 99% of all healthy individuals will have a labelling percentage above 45%, while at most 1% of the cancer patients will show a percentage above 30%. It has been shown that the procedure for assessment of the labelling results can be done either by a machine-aided procedure or by a visual assessment procedure (Davina et al., 1985b).

In the present study, the concanavalin A-peroxidase (ConA-HRP) labelling method has also been applied to cervical cell suspensions from patients with precancerous and cancerous lesions. The study addressed the determination of the labelling percentage of normal flattened epithelial cells in healthy individuals and in case of mild, moderate, and severe dysplasia, carcinoma in

situ, and squamous cell carcinoma of the uterine cervix. In addition, the course of the labelling results (alteration in labelling percentage) during follow-up of patients with a persisting epithelial abnormality and of patients treated for a cervical abnormality was determined. Also the change in labelling percentage caused by therapeutic intervention was studied. Finally, the diagnostic accuracy of the ConA-HRP labelling method for the detection of cancerous and precancerous lesions of the uterine cervix was compared with that of the Papanicolaou method.

MATERIALS AND METHODS

Cytopathological material

Cervical cells were collected on one or more times from healthy individuals (n=40), and from patients with a mild (n=10), moderate (n=15), or severe (n=28) dysplasia, from patients with a carcinoma in situ (n=24), and from patients with a squamous cell carcinoma (n=10). Also samples from patients after conservative treatment (ablatio, conisation) or by way of uterine extirpation for severe dysplasia (n=17), carcinoma in situ (n=15), or squamous cell carcinoma (n=7) were studied. A small number of the latter patients form part of the former patients.

For the follow-up study, samples were used from part of all the above mentioned patients on two different occassions during their disease, or on two different occasions after treatment. The aim of this follow-up study was to investigate whether the labelling percentage is consistent in case of an unchanged stage of disease. In order to investigate whether treatment results in a change of the labelling percentage, from part of the above mentioned patients samples were taken before and after therapeutic intervention. Sampling of these follow-up groups of patients was achieved by taking all patients who returned to the clinic in a period of half a year (October 1983-March 1984) after their initial consultation. The period between the two times varied from 2 to 10 months.

The lesions were classified by repeated Papanicolaou cytology, followed by histological confirmation in cases of moderate and severe dysplasia, carcinoma in situ, and squamous cell carcinoma. Final classification was based on histological examination.

ConA-HRP labelling

Full details about the preparation of cervical cell suspensions and about the labelling procedure have been reported previously (Davina et al., 1985a). The quantification of the labelling results, has been carried out by one observer using the visual assessment procedure (Davina et al., 1985b).

Statistical analysis

Differences in labelling results between the healthy individuals and the patients with mild, moderate, or severe dysplasia, and patients with carcinoma in situ or squamous cell carcinoma were studied. One sample per patient was used and a one-way analysis of variance, followed by simultaneous pairwise comparisons according to Scheffé, was applied. The Student's test for paired samples was used to examine the difference between the labelling percentages at two different points in time either during the disease or after treatment. The same test was used to examine the differences between labelling percentages before and after treatment. The Pearson test was used to analyse the correlation between the labelling percentage of treated patients and the time span after treatment. This correlation was studied in patients taken at an arbitrary point in a period of 2-48 months after treatment. For the control group a lower bound (one-sided guaranteed coverage lower tolerance limit) for the 1%-point of the distribution of the labelling percentage has been established. Below this limit there are probably at most 1% of the healthy individuals. The chosen level of confidence was 90%, and a normal distribution was supposed. In an analogous way an upper bound has been established for the group of cancer patients. The difference in detection rate between the ConA-HRP method and the Papanicolaou method was examined by means of Mc Nemar's tests.

RESULTS

The distribution of percentages of ConA-HRP labelled flattened cells from individual healthy controls and from individual patients with precancerous and cancerous lesions of the uterine cervix is shown in figure 1. When more specimens of the same patient were available, one specimen was chosen at random. Table 1 shows the mean labelling percentages with the standard deviation of each group. From the data presented, it can be seen that the highest mean of percentages of ConA-HRP labelled flattened epithelial cells, is found in the normal group. The lowest mean is found in patients with

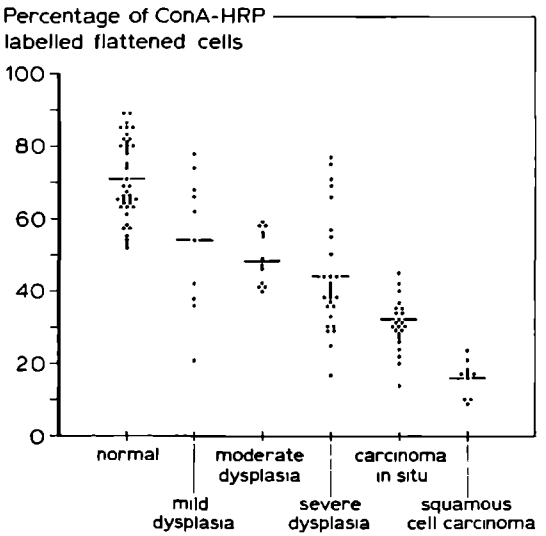

Figure 1.
Distribution of percentages of ConA-HRP labelled flattened cells from healthy individuals and from patients with precancerous and cancerous lesions of the uterine cervix; the horizontal bar indicates the mean of the group.

squamous cell carcinoma. Moreover, the labelling percentage decreases with increasing atypia. By one-way analysis of variance, the hypothesis of equal means was rejected ($p < 0.001$). Pairwise multiple comparisons according to Scheffé showed that this was mainly caused by a higher mean for the control group as compared to each other group ($p \leq 0.02$), and by a lower mean for the squamous cell carcinoma group as compared to each other group ($p \leq 0.05$). Also the mean labelling percentage for the carcinoma in situ group was significantly lower than the mean percentage for the moderate dysplastic group ($p=0.02$), and almost significantly lower than the mean percentage for the group with severe dysplasia ($p=0.07$). No significant differences were found between the three groups with a dysplasia ($p \geq 0.49$).

Table 1.
Mean percentages of ConA-HRP labelled flattened cells from healthy individuals and from patients with precancerous and cancerous lesions of the uterine cervix.

histological diagnosis	number of women	mean (±s.d.)
normal	40	71 (±11)
dysplasia mild	10	54 (±19)
moderate	15	48 (±13)
severe	28	44 (±16)
carcinoma in situ	24	32 (±11)
squamous cell carcinoma	10	16 (± 5)

A one-sided guaranteed coverage lower tolerance limit has been determined with respect to the 1%-point of the distribution of the labelling percentage for healthy individuals. Supposing a normal distribution and using a level of confidence of 90%, this limit is 40%, when the visual assessment procedure has been used. This means that probably at most 1% of all healthy individuals possesses a labelling percentage below 40%. The upper tolerance limit, above which are probably at most 1% of the cancer patients, was 33%.

Using the ConA-HRP labelling method with 40% as a discriminative level, it is seen from figure 1 that the method completely discriminates between all healthy individuals and squamous cell carcinoma patients under consideration. Table 2 shows a comparison of detection rates of the ConA-HRP labelling method and the Papanicolaou method for cancerous and precancerous lesions of the uterine cervix. From this table it follows that the Papanicolaou method is (nearly) significantly better suited for the detection of patients with a dysplastic lesion. Although the ConA-HRP labelling method gives higher detection rates for patients with a carcinoma in situ or squamous cell carcinoma compared with the Papanicolaou method, no significant differences could be found.

From some of the above mentioned patients, cell material was collected at two different times, whereas no therapeutic intervention was applied and whereas the Papanicolaou classification remained unchanged. The labelling results of these patients at two different times are shown in table 3. For each group a

Table 2.
Comparison of detection rates of the ConA-HRP labelling method and the Papanicolaou method for healthy individuals and for precancerous and cancerous lesions of the uterine cervix; p-values indicate the results of testing the hypothesis of equal probability of detection by means of the Mc Nemar's test.

histological diagnosis	number of women	ConA-HRP	Papanicolaou	p-value
normal	40	0	0	
dysplasia mild	10	0.30	0.90	$0.05 < p \leq 0.10$
moderate	15	0.13	0.80	< 0.01
severe	28	0.50	0.82	$0.01 < p \leq 0.20$
carcinoma in situ	24	0.88	0.83	> 0.10
squamous cell carcinoma	10	1.00	0.60	> 0.10

Table 3.
Mean percentages of ConA-HRP labelled flattened cells from patients at two subsequent times during their disease; p-values indicate the results of testing the hypothesis of no change by means of the paired Student's test.

histological diagnosis	number of women	mean (±s.d.)		p-value
		first time	second time	
moderate dysplasia	5	47 (±20)	43 (±10)	0.72
severe dysplasia	12	48 (±17)	41 (±14)	0.26
carcinoma in situ	7	32 (± 9)	31 (± 5)	0.82
squamous cell carcinoma	5	17 (± 4)	15 (± 6)	0.50

lower mean was observed at the second time. However, it appeared according to the paired Student's test, that the labelling percentage of the second time did not significantly differ from the percentage of the first time. Neither a significant difference was found after combining the groups (p=0.20).

Some of the patients have been treated for severe dysplasia, carcinoma in situ, and squamous cell carcinoma. In table 4 the mean labelling percentages from these patient groups are shown before and after treatment. It can be seen that the labelling percentage after treatment is higher than the percentage before treatment. These differences appeared to be significant according to the paired Student's test (p=0.01) for both the combined severe dysplasia/carcinoma in situ group, and for the squamous cell carcinoma group.

Other patients who were also treated for severe dysplasia, carcinoma in situ, and squamous cell carcinoma, were then considered in order to examine the labelling percentage after treatment. These groups of patients were augmented by similar patients mentioned in table 4. When for a patient specimens from

Table 4.
Mean percentages of ConA-HRP labelled flattened cells from patients before and after treatment for precancerous and cancerous lesions of the uterine cervix; p-values indicate the results of testing the hypothesis of no change by means of the paired Student's test.

histological diagnosis	number of women	mean (±s.d.)		p-value
		pretreatment	posttreatment	
severe dysplasia/ /carcinoma in situ	9	39 (±12)	61 (±15)	0.01
squamous cell carcinoma	4	15 (± 7)	74 (±20)	0.01

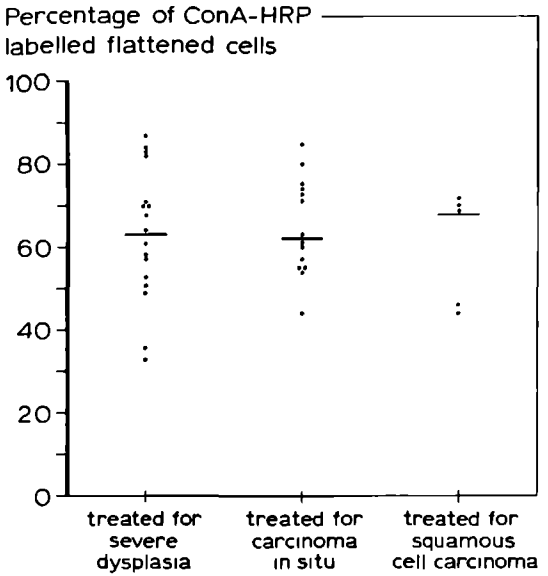

Figure 2.
Distribution of percentages of ConA-HRP labelled flattened cells from patients after conservative treatment for precancerous and cancerous lesions of the uterine cervix, the horizontal bar indicates the mean of the group.

several days were available, one of them was chosen at random. Figure 2 shows the distribution of the labelling percentages of the individual patients treated for severe dysplasia, carcinoma in situ, or squamous cell carcinoma. Table 5 presents the mean labelling percentages with respect to the three treated lesions. They are about the same level as the percentage of healthy individuals.

In the group of patients that were treated for severe dysplasia, no significant correlation ($p=0.67$) could be established between the labelling percentage and the time span after treatment (Pearson correlation coefficient=0.11). On the other hand, patients treated for carcinoma in situ showed a significantly higher labelling percentage ($p=0.04$) with an increasing

Table 5.
Mean percentages of ConA-HRP labelled flattened cells from patients treated for precancerous and cancerous lesions of the uterine cervix.

after treatment for	number of women	mean (±s.d.)
severe dysplasia	17	63 (±16)
carcinoma in situ	15	62 (±14)
squamous cell carcinoma	7	68 (±18)

time span post-treatment (Pearson correlation coefficient=0.53). The Pearson correlation coefficient for patients treated for squamous cell carcinoma amounted 0.65, but it may be that the number of patients was to small to be able to determine a significant association (p=0.11).

In addition, the per patient change in labelling percentage after treatment was studied. The labelling percentages of 16 patients taken on two occasions were compared. The period between the two times varied from 2 to 10 months. The Student's test for paired observations did not reveal a significant increase (means respectively 67% and 70%, p=0.54).

DISCUSSION

It has been shown in previous studies that the cell surface of normal cells may undergo alterations before or during the development of (pre)malignancy in the uterine cervix (Davina et al., 1984). This datum has been used to develop a ConA-HRP labelling procedure which allows the discrimination between healthy individuals without any known epithelial abnormality and patients with a histologically confirmed squamous cell carcinoma (Davina et al., 1985a; Davina et al., 1985b). In this study, the ConA-HRP labelling procedure has also been applied to cervical cells from patients with a mild, moderate, or severe dysplasia, and from patients with a carcinoma in situ.

The follow-up study shows that the labelling percentage of patients of a disease group does not alter clearly. Moreover, the follow-up study shows that patients after treatment possess the normal high labelling percentage again with the exception of a small number of patients. The cell samples of these patients were not taken just after treatment. So it is not impossible that these exceptions may represent patients with a higher risk of recurrence.

The relative frequency of false negatives in the group of carcinoma in situ patients (labelling percentage above 40%) is very low (12%). In the group of patients with severe dysplasia the relative frequency of false negatives appears to be 50%. According to Fisher's exact test for a 2x2-table there is a significant difference ($p<0.01$). This difference is striking because other approaches do not offer a possibility to clearly discriminate severe dysplasia from carcinoma in situ, not only cytomorphologically and histologically, but also cytogenetically (Wakonig-Vaartja and Hughes, 1965), immunologically

(Saksela and Meyer, 1973), and immunocytochemically (McDicken and Rainey, 1983). It is possible that the aforementioned approaches "overdiagnose" severe dysplasia to carcinoma in situ. Our results indicate that a more optimal discrimination is possible. These results may reflect the occurrence of an essential alteration during the transition from severe dysplasia to carcinoma in situ. However, the biology of the transition between both stages is not understood.

The current cytological screening method of cervical cancer according to Papanicolaou is one of the best cancer diagnostic tests currently available. It has aided in the drastic reduction of deaths from cervical cancer. Our studies have shown that cytochemical cell surface analysis may also be a suitable screening method in terms of specificity and sensitivity, objectivity and rapidity. The ConA-HRP labelling method has the advantage that an abundant number of cells is available for detection, in contrast to the frequently inadequate number of (pre)neoplastic cells which may cause sampling and screening errors. Whereas labelled and unlabelled cells show a distinct difference in labelling intensity, the (pre)neoplastic cells show a continuous transition from a less to a more atypical character, which causes a less objective cytological diagnosis. The ConA-HRP labelling method allows the complete discrimination between healthy women and squamous cell carcinoma patients. This labelling method detected proportionately more cancer patients, compared with the Papanicolaou test. However, this difference was not significant. On the other hand the dectection rate is lower for dysplasia patients. With regard to the patients with a carcinoma in situ, the ConA-HRP method seems to score better compared with literature data on the occurrence of false-negative findings using the Papanicolaou method (cf. Coppleson and Brown, 1974). The ConA-HRP method is a simple procedure with little danger of technical errors. In principal, the method allows flow cytometrical treatment of cell samples which might offer a more rapid and more accurate application of the method.

Our results seem to indicate that patients with a labelling percentage below 40% and who have a negative Papanicolaou cytology, need continual surveillance. This is especially so knowing that not all cervical cancers pass through a long-standing phase of premalignancy (Ashley, 1966; Hakama and Penttinen, 1981; Coppleson and Brown, 1981), and that not all cases with

distinct dysplasia and carcinoma in situ advance to invasive cancer (Patten, 1978; Peterson, 1956). It may be that the continual application of the ConA-HRP labelling method can predict the future behavior of a lesion because all cancer patients are characterized by a low labelling percentage, and because a gradual decrease of the labelling percentage exists with increasing atypia. The prognostic value can be extended by application of other cytochemical labelling techniques as for instance labelling of antigenic determinants. Valet et al. (1981) have shown an improved sensitivity in the detection of abnormal cells from cervical cancers. They have simultaneously measured antigenic determinants of carcino-embryonic antigen (CEA) and epithelial membrane antigen (EMA) on the cell surface together with cell volume and cellular DNA. Boon et al. (1982) have shown CEA positivity in squamous and in malignant exfoliated epithelial cells in sputum from patients with carcinoma of the lung. Their possible prognostic significance was found in the CEA-positivity of benign squamous cells from high-risk patients without clinical evidence of neoplasm. Wagner and Noltenius (1983) have suggested that cervical cell suspensions without a positive erythrocyte adherence test might not be related to cervical carcinoma until the rosette test becomes positive. Das and Roy-Chowdhury (1981) have stated that the glycogen accumulation in cervical epithelial cells can possibly serve as a useful tool in the early detection of recurrence.

In conclusion, we consider the ConA-HRP labelling method as a supplementary method to the Papanicolaou method in the screening for the early detection of cervical cancer. The ConA-HRP labelling method reduces the effect of the sampling and screening errors of the Papanicolaou method, and it allows a more objective cytological diagnosis. In addition, the ConA-HRP labelling method may possess prognostic significance.

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Summary

CELL SURFACE CHARACTERISTICS AND THE DETECTION OF (PRE)MALIGNANCY OF THE HUMAN UTERINE CERVIX

This thesis describes the results of investigations into alterations in the cell surface of epithelial cells in the case of neoplasia of the human uterine cervix. The plasma membrane-cell surface complex is of decisive significance to the social behavior of the cell and its alterations such as neoplasia, tumor invasion and metastasis. The investigations have been undertaken to improve the screening for the early detection of neoplastic transformation. The Papanicolaou method which is in general use, is liable to sampling and screening errors as a consequence of the small number of neoplastic cells present in a smear. In addition, the conventionaly cytological criteria in the Papanicolaou method do not always make the discrimination of non-neoplastic from neoplastic changes possible. The present study is aimed at a reduction of these errors to allow a more reliable cytological diagnosis. The plasma membrane-cell surface complex of the morphologically normal epithelial cells has been, in particular, the subject of investigation. An abundant number of these cells is available. Moreover, it is to be expected that, initially the cell surface of normal cells will react to factors which cause or further the development of neoplastic alterations such as viral infections (general introduction).

In chapter 1, the surface of squamous epithelial cells and its differentiation were studied with the scanning electron microscope (SEM). The purpose was to determine whether or not ultrastructural characteristics could be used to discriminate normal from neoplastic cells. This information is relevant when applying other methods of cell surface research such as surface labelling. Use was made of a developed "stripping" technique and of a freeze-fracture technique. After SEM-study, the biopsies were also examined light microscopically. The size and shape of the cells and the length and width of the cellular processes change gradually during the cell migration from the basal to the exfoliating layer of the normal non-keratinizing stratified squamous epithelium. As a result of the differentiation process the flattened cells acquire a characteristic pattern of microridges and globular microvilli,

and crests and furrows on their surface. On the luminal cell side microridges and crests are observed, whereas globular microvilli and furrows occur on the basal side. Microridges and crests interdigitate respectively with globular microvilli and furrows present on the basal side of the overlying cell. Crests and furrows delineate regions on the cell surface in which variable microridge- and microvillus patterns appear.

In chapter 2, the question of whether the surface of normal and (pre)malignant cells is characterized by the appearance or disappearance of SEM cell surface properties was studied. The usefulness of SEM cell surface properties for diagnosis of cancer was reviewed in human and in animal tumors, both in vivo and in vitro. It appeared that malignant cells constitute a very heterogenous group. Whereas cancer cells cannot be defined as cells totally lacking differentiation, the statement that progressive severity of cervical intraepithelial neoplasia can be characterized as an increasing impairment of cell and tissue differentiation seems to hold true. Based partially on our own observations with the SEM it can be said that during the development of an invasive intraepithelial cancer fewer and fewer microridges and more and more microvilli can be seen on the cell surface. However, microvilli cannot be considered to be indicative of abnormality as they are also found on non-malignant cells. In addition, microridges do not imply normality as they also occur on some cancer cells. The presence of long and pleomorphic microvilli is also not pathognomic for the tumor process. So cell surface characteristics as observed with conventional SEM techniques do not appear to be useful as markers of neoplastic transformation.

In chapter 3, the question of whether cytochemical cell surface alterations occur during normal differentiation and neoplastic transformation of the cervical epithelium was investigated. In this transmission electron microscopical study of biopsy tissues, tannic acid (TA) was used for visualization of alterations in the carbohydrate-rich glycocalyx of the cell surface. For objective determination of the TA-binding results, computer-controlled microdensitometry was applied. While in normal epithelium there is distinct TA binding in four to five cell layers of the deep intermediate zone, cells of carcinoma in situ and invasive cancer lesions lack TA binding. In moderate dysplasia an intermediate pattern is found. Deep intermediate cells in areas bordering the carcinoma in situ lesions do not show any binding, although their ultrastructure cannot be distinguished from

similar cells in normal tissue. The results indicate that an alteration in the carbohydrate-rich glycocalyx occurs during normal differentiation of the epithelium, which is disturbed in the presence of a (pre)malignant lesion.

In chapter 4, it was studied whether the cytochemical alterations of the glycocalyx could also be demonstrated on exfoliating cells of the uterine cervix by lectin binding of the cell surface. Labelling of exfoliated cells was carried out using concanavalin A-horseradish peroxidase (ConA-HRP) as a label. Although there is some variation in labelling intensity there is a distinct difference between the labelling intensity of labelled and that of unlabelled cells. Quantification of these labelling results was achieved using a light microscope equipped with a computerized video system. Both normal and cancer specimens contain a fairly constant fraction of labelled flattened epithelial cells with a microscopically normal appearance. A significant difference exists between this percentage of labelled cells from the healthy individuals and that of the patients with a histologically confirmed squamous cell carcinoma. A high labelling percentage has been found in the control group (mean 73%, s.d. 10), and a low percentage in the cancer group (mean 15%, s.d. 4). Influences related to the phase of the menstrual cycle, the use of oral contraceptives, or the postmenopause could not be established. Statistical analysis of the available data revealed that the specificity as well as the sensitivity of the ConA-HRP labelling method in the detection of cancer patients is at least 99%, when the labelling percentage of 45% is chosen as critical value. It is concluded that the ConA-HRP labelling of normal epithelial cells seems to provide a more reliable diagnosis of cervical cancer. It is not liable to sampling and screening errors such as occur with the Papanicolaou method.

In chapter 5, it was investigated whether the labelling percentage can also be determined without the more advanced and expensive equipment. This would allow the use of the ConA-HRP labelling method in any cytopathological laboratory. In particular the interobserver and intraobserver variations were studied. A statistical analysis of the results showed that the ConA-HRP method using the assessment procedure with a light microscope only has a similar high discriminatory capacity in the detection of cancer patients to that which has been established for the machine-aided assessment procedure. Although the former method is accompanied by slight variation, it can be used as a routine method.

In chapter 6, the feasibility of the ConA-HRP labelling method was determined for the detection of patients with a premalignant lesion. The results show that the ConA-HRP labelling percentage of apparently normal flattened epithelial cells in smears from precancerous lesions gradually decreases with increasing atypia of the epithelium. In a follow-up study, it was found that the labelling percentage does not alter when the stage of disease remains unchanged. A re-establishment of the normal ConA-HRP labelling result often appears after conservative treatment of the precancerous or cancerous lesion.

Based on the studies described in chapters 4, 5 and 6, it is concluded that the ConA-HRP labelling method can be considered as an important supplementary method to the Papanicolaou method for an improved detection of cervical cancer. It reduces the sampling and screening errors of the Papanicolaou method, and it allows a more objective semi-quantitative cytological diagnosis. In addition, when the course of the ConA-HRP labelling percentage of a patient is followed longitudinally, the method may contribute to the prediction of the future behavior of a cervical intraepithelial neoplastic lesion.

Samenvatting

KENMERKEN VAN HET CELOPPERVLAK EN DE OPSPORING VAN (PRE)MALIGNE AFWIJKINGEN VAN DE MENSELIJKE BAARMOEDERHALS

In dit proefschrift worden de resultaten beschreven van een onderzoek naar de veranderingen aan het celoppervlak van epitheelcellen van de menselijke baarmoederhals in geval van neoplasie. Bij het sociaal normale gedrag van cellen in een epitheel en bij veranderingen daarin zoals bij neoplasie, tumorinvasie of metastasering, wordt een belangrijke rol toegeschreven aan het plasmamembraan-celoppervlakte complex. Het hier beschreven onderzoek werd verricht met het doel te komen tot een verbeterde methode voor vroege opsporing van neoplastische veranderingen. Bij de algemeen gebruikte cytologische methode volgens Papanicolaou bestaat het risico van onjuiste beoordelingen die samenhangen met de techniek van afnemen van celmateriaal bij de patient ("sampling error"), of die kunnen optreden bij het beoordelen van het uitstrijkpreparaat ("screening error"). Bovendien maken de gebruikelijke criteria zoals gehanteerd bij de Papanicolaou-methode, het niet altijd mogelijk om neoplastische en niet-neoplastische veranderingen van elkaar te onderscheiden. De beoordelingsfouten waarvan hierboven sprake is, zijn onder meer het gevolg van het kleine aantal neoplastische cellen dat normaliter in het gewonnen celmateriaal voorkomt. Het in dit proefschrift beschreven onderzoek richt zich vooral op de mogelijkheid tot het reduceren van deze fouten, zodat een meer betrouwbare cytologische diagnose mogelijk wordt. In het bijzonder is het plasmamembraan-celoppervlakte complex van epitheelcellen met een normale morfologie bestudeerd. Het is reeel te veronderstellen, dat in de eerste plaats het celoppervlak van normale cellen zal reageren op factoren die de ontwikkeling van neoplastische veranderingen veroorzaken of bevorderen zoals virale infecties. Bovendien zijn normale cellen in ruime mate aanwezig in een cytologisch uitstrijk-preparaat (zie "General Introduction").

In hoofdstuk 1 wordt een scanning elektronenmicroscopisch (SEM) onderzoek beschreven naar het oppervlakte-topografisch patroon van normale plaveisel-epitheelcellen en naar de differentiatie daarvan. Het doel is na te gaan, in hoeverre ultrastructurele kenmerken kunnen worden gevonden die normale cellen van neoplastische cellen kunnen onderscheiden. Deze informatie is ook van

belang bij de interpretatie van resultaten verkregen met andere methoden van celoppervlakte-onderzoek zoals oppervlaktelabelling. Er is gebruik gemaakt van een speciaal ontwikkelde "stripping"-techniek en van de vriesbreek-techniek. Na de bestudering in de SEM zijn de onderzochte biopten ook lichtmicroscopisch gecontroleerd. Tijdens migratie van de cellen van de basale naar de exfolierende laag van het meerlagig niet-verhoornend plaveiselepitheel, treedt er een geleidelijke verandering op in de grootte en de vorm van de cellen, die gepaard gaat met veranderingen in de lengte en breedte van de celoppervlakte-uitstulpingen. Dit differentiatieproces resulteert in het verschijnen van een karakteristiek patroon van microplooien (microridges), globulaire microvilli, kammen (crests) en groeven (furrows) op het oppervlak van de afgeplatte plaveiselepitheelcellen. Op de naar het lumen gekeerde celzijde worden microridges en crests waargenomen, terwijl op de naar de basaal membraan gekeerde celzijde globulaire microvilli en furrows worden aangetroffen. Het is gebleken, dat zowel de microridges en globulaire microvilli, als de crests en furrows interdigiteren waar de bovenzijde van de ene cel grenst aan de onderzijde van de erboven gelegen cel. De crests en furrows bakenen gebieden op het celoppervlak af die onderling een variabel patroon van microridges en microvilli vertonen.

In hoofdstuk 2 is onder meer in de vorm van een literatuurstudie in meer algemene zin nagegaan of het oppervlak van normale ten opzichte van (pre)maligne cellen kan worden gekarakteriseerd door het optreden c.q. ontbreken van specifieke celoppervlakte kenmerken. Daartoe is een overzicht gegeven van de diagnostische bruikbaarheid van SEM-structuren van het celoppervlak bij een verscheidenheid van menselijke en dierlijke tumoren, die in vivo en/of in vitro bestudeerd zijn. Daarbij is gebleken, dat maligne cellen een erg heterogene groep vormen. Hoewel dergelijke cellen niet kunnen worden gedefinieerd als cellen die geen differentiatie ondergaan, kan toch worden vastgesteld, dat bij toenemende ernst van een cervicale intra-epitheliale neoplasie een steeds ernstiger gestoorde differentiatie van de cellen en het weefsel optreedt. Bij de ontwikkeling van een invasief plaveiselcelcarcinoom worden aan het celoppervlak steeds minder microridges en steeds meer microvilli waargenomen. Mede op grond van eigen onderzoek kan geconcludeerd worden dat microvilli niet kunnen worden beschouwd als uiting van een abnormale c.q. maligne celgroei, omdat dergelijke structuren ook worden aangetroffen op het oppervlak van niet-maligne cellen. Omgekeerd impliceert het voorkomen van microridges niet, dat een cel normaal is, omdat ook maligne

cellen microridges kunnen bezitten. Evenzo blijkt de aanwezigheid van lange en veelvormige microvilli niet kenmerkend te zijn voor het bestaan van een maligne groeiproces. Geconcludeerd wordt, dat celoppervlakte-kenmerken zoals deze met conventionele SEM-technieken kunnen worden waargenomen, niet bruikbaar zijn als aanwijzing voor het bestaan van neoplastische veranderingen in cellen van het cervicale epitheel.

In hoofdstuk 3 zijn de resultaten weergegeven van een onderzoek naar cytochemische aanwijzingen voor een veranderend celoppervlak tijdens de normale differentiatie en de maligne ontaarding van het epitheel van de baarmoederhals. Om redenen van praktische aard is gebruik gemaakt van een tanninekleuring gevolgd door transmissie elektronenmicroscopisch onderzoek van bioptweefsel om veranderingen in de koolhydraatrijke "buitenzijde" of glycocalyx van de epitheelcellen zichtbaar te maken. Voor een objectieve registratie van de tanninezuur-binding is gebruik gemaakt van computer gestuurde microdensitometrie. Vastgesteld kon worden, dat in het normale epitheel de cellen van het dieper gelegen deel van de intermediaire zone een relatief hoge affiniteit hebben voor tanninezuur; in andere delen van het epitheel is de binding niet duidelijk waarneembaar. Cellen van een matige dysplasie tonen een variabel kleuringspatroon met tanninezuur. Binding van tanninezuur kon niet worden vastgesteld bij de cellen van een carcinoma in situ en een invasief carcinoom. Ook de diep-intermediaire cellen in gebieden die grenzen aan een carcinoma in situ lesie, binden geen tanninezuur, hoewel er geen ultrastructurele aanwijzingen zijn om dergelijke cellen te onderscheiden van gelijksoortige cellen in normaal weefsel. Samenvattend wijzen de resultaten erop, dat de glycocalyx aan verandering onderhevig is tijdens de normale differentiatie van het epitheel, terwijl voorts het normale ontwikkelingspatroon gestoord is in geval van de aanwezigheid van een (pre)maligne lesie.

In hoofdstuk 4 worden de resultaten vermeld van een onderzoek naar de cytochemische veranderingen van de glycocalyx bij de exfolierende cellen van het baarmoederhalsepitheel door de binding van lectinen aan het celoppervlak te onderzoeken. De cellen werden daartoe gelabeld met concanavaline A-mierikswortel peroxydase (ConA-HRP). Hoewel de labelintensiteit tot op zekere hoogte kan varieren, is er toch een duidelijk verschil tussen de gelabelde en niet gelabelde cellen aanwezig. Dit verschil is gemakkelijk objectief vast te stellen met behulp van een lichtmicroscoop uitgerust met een gecomputerizeerd videosysteem. Het blijkt, dat elke patiente in de tijd een

tamelijk constante fractie gelabelde afgeplatte epitheelcellen met een normale morfologie bezit. Uit de bepaling van dit percentage gelabelde afgeplatte epitheelcellen bij gezonde vrouwen en bij patienten met een histologisch bevestigd plaveisel-celcarcinoom van de baarmoederhals blijkt, dat er een significant verschil bestaat tussen de gezonde groep (gemiddelde 73%, standaardafwijking 10) en de kankergroep (gemiddelde 15%, standaardafwijking 4). Het percentage gelabelde cellen blijkt niet te veranderen onder invloed van de fase in de menstruatiecyclus, het gebruik van orale contraceptiva of de post-menopauze. Statistische bewerking van de beschikbare gegevens leert, dat zowel de specificiteit als de sensitiviteit van de ConA-HRP labellingsmethode voor het detecteren van kankerpatienten ten minste 99% bedraagt, wanneer een labelingspercentage van 45% als kritische waarde wordt gekozen. Er kan worden geconcludeerd, dat ConA-HRP labelling van normale plaveiselepitheelcellen als methode voor het detecteren van kankerpatienten een hoge diagnostische betrouwbaarheid lijkt te kunnen bieden. Met name worden beoordelingsfouten zoals bij de Papanicolaou-methode voorkomen, vermeden.

In hoofdstuk 5 is onderzocht of het vaststellen van het percentage gelabelde cellen ook mogelijk is zonder gebruik van de geavanceerde en kostbare meetapparatuur. Dit onderzoek is uitgevoerd om na te gaan of de ConA-HRP labellingsmethode ook in het routinematig cytopathologisch onderzoek kan worden toegepast. In het bijzonder is de variatie onderzocht welke samenhangt met de onderzoeker en het tijdstip van onderzoek (interobserver- en intraobserver-variatie). Statistische bewerking van de resultaten leert, dat ook in geval van de meer subjectieve lichtmicroscopische beoordeling de ConA-HRP methode eenzelfde hoog onderscheidend vermogen bezit bij de opsporing van kankerpatienten, zoals eerder is vastgesteld voor de objectieve registratieprocedure. Ook al bezit de eerstgenoemde beoordelingsprocedure een geringe interobserver en intraobserver variatie, deze procedure is toch geschikt om routinematig te worden toegepast.

In hoofdstuk 6 is het ConA-HRP onderzoek uitgebreid tot patienten met een premalignе afwijking. De resultaten tonen, dat bij het ernstiger worden van de epitheliale afwijking het labelingspercentage van normale polygonale cellen geleidelijk afneemt. Tevens blijkt uit vervolg-onderzoek dat als er geen progressie of regressie is in de ernst van de afwijking, het percentage gelabelde cellen niet verandert. Tot slot is vastgesteld, dat wanneer de patiente behandeld is voor de neoplastische afwijking met behoud van de cervix,

in de meeste gevallen weer een normaal hoog labellingspercentage wordt aangetroffen.

Op grond van de onderzoeken vermeld is de hoofdstukken 4, 5 en 6 wordt geconcludeerd, dat de ConA-HRP labellingsmethode beschouwd lijkt te kunnen worden als een belangrijke aanvulling op de Papanicolaou-methode. Hierdoor wordt een verbeterde opsporing van een maligne ontaardingsproces in de baarmoederhals mogelijk gemaakt. De ConA-HRP methode reduceert het aantal beoordelingsfouten welke de Papanicolaou-methode kent. De methode maakt ook een meer objectieve en semi-kwantitatieve cytologische diagnose mogelijk. Wanneer het verloop van het percentage gelabelde cellen van een patiente in de tijd wordt gevolgd, levert de ConA-HRP methode mogelijk ook een bijdrage aan de voorspelling van het toekomstig gedrag van een cervicale intra-epitheliale afwijking.

Curriculum Vitae

Op 14 november 1951 ben ik als Oldenzaler in de gemeente Losser geboren. Het gymnasium-B diploma werd in 1970 aan het Twents Carmellyceum te Oldenzaal behaald. Daarna ben ik met de studie Biologie begonnen. De landelijke plaatsingscommissie willigde mijn wens in en plaatste mij aan de Landbouw Hogeschool te Wageningen. Het propaedeutisch examen werd afgelegd in juni 1971 en het kandidaatsexamen in januari 1974. In 1974 heb ik ook mijn eerste graads bevoegdheid behaald voor het biologie-onderwijs. Van 1973 tot en met 1976 ben ik elk jaar gedurende twee maanden als studentassistent verbonden geweest aan de sectie histologie van de vakgroep Experimentele Diermorfologie en Celbiologie. Vanaf mijn doctoraalstudie heb ik met veel enthousiasme deelgenomen aan activiteiten en het bestuur van het Wageningse Studenten-pastoraat. In januari 1977 studeerde ik cum laude af. De hoofdvakken waren celbiologie (Mevr.Prof.Dr. L.P.M. Timmermans) en dierfysiologie (Prof.Dr. L.M. Schoonhoven). Van 1977 tot juli 1979 ben ik in het kader van de tewerkstellingsregeling voor erkend gewetensbezwaarden militaire dienst als wetenschappelijk onderzoeker werkzaam geweest binnen het project "De afweer-reactie in de darm van karperachtigen in relatie tot de aard van het voedsel". Dit onderzoek werd uitgevoerd aan de vakgroep Experimentele Diermorfologie en Celbiologie van de Landbouw Hogeschool onder leiding van Mevr.Prof.Dr. L.P.M. Timmermans. In augustus 1979 is het onderzoek gestart, dat geresulteerd heeft in dit proefschrift. Het betrof een samenwerkingsverband met de afdelingen Submicroscopische Morfologie (promotor Prof.Dr. A.M. Stadhouders), Pathologische Anatomie (promotor Prof.Dr. U.J.G.M. van Haelst), en Obstetrie en Gynaecologie (co-referent Dr. P. Kenemans) van de subfaculteit Geneeskunde van de Katholieke Universiteit Nijmegen. Het onderzoeksproject, dat als titel voerde "Celoppervlakte-organisatie van normale en maligne epitheelcellen van de humane cervix uteri" werd gesubsidieerd door het Koningin Wilhelmina Fonds. Deze nederlandse organisatie voor de kankerbestrijding subsidieerde ook mijn deelname aan internationale congressen in Clausthal-Zellerfeld (West Duitsland), Dallas (Texas, U.S.A.), Nijmegen, Noordwijkerhout en Tokio (Japan). In februari 1984 is dit onderzoek officieel afgesloten. Vanaf die datum heb ik in een volontairscontract de publicitaire activiteiten afgerond, en ben ik op een creatieve manier bezig met het zoeken naar een volgende boeiende bezigheid.

Stellingen behorend bij het proefschrift:
"CELL SURFACE CHARACTERISTICS AND THE DETECTION OF (PRE)MALIGNANCY OF THE HUMAN UTERINE CERVIX"

J.H.M. Davina, 13 december 1984

I

Kenmerken van het oppervlak van de epitheelcellen van de baarmoederhals zijn niet alleen afhankelijk van de cel zelf maar ook van de wisselwerking tussen de cellen. (dit proefschrift)

II

Neoplastische veranderingen van de epitheelcellen van de baarmoederhals kunnen met conventionele scanning elektronenmicroscopische technieken niet worden vastgesteld. (dit proefschrift)

III

Voor het opsporen van baarmoederhalskanker dient cytochemisch celoppervlakte-onderzoek te worden verricht. (dit proefschrift)

IV

In geval van een negatieve uitslag van het cytologisch onderzoek volgens Papanicolaou kan labelling van morfologisch normale plaveiselepitheelcellen met concanavaline A aangeven bij welke vrouwen het onderzoek met een verhoogde frequentie herhaald moet worden. (dit proefschrift)

V

Voor de succesvolle behandeling van recidiverend cervixcarcinoom met bromocriptine ontbreekt een wetenschappelijke verklaring dit in tegenstelling tot de situatie bij amenorrhoea en galactorrhoea.

Rolland, R., et al., Clin. Endocrinol., 3: 155-165, 1974.
Guthrie, D., Brit. J. Obstet. Gynaecol., 89: 853-855, 1982.

VI

Voor een verbeterde classificatie in de tumordiagnostiek zal behalve licht- en elektronenmicroscopisch morfologisch onderzoek ook steeds meer gebruik moeten worden gemaakt van (immuno)cytochemische methoden.

VII

Hoewel glucocorticoïd-therapie af te raden is voor de behandeling van cerebrale malaria, kan een dergelijke behandeling wel preventief werken.

Warrell, D.A., et al., N. Engl. J. Med., 306: 313-319, 1982.
Eling, W.M.C., Fortschr. Zool., 27· 141-155, 1982.

VIII

PAS-positieve granulocyten bij vissen en mestcellen bij zoogdieren zijn homoloog.

Davina, J.H.M., et al., In: J.D. Horton (ed.), Development and differentiation of vertebrate lymphocytes. Elsev./North-Holl. Biomed. Pr., pp. 129-140, 1980.
Ali, N.M., Experientia, 40: 197-199, 1984

IX

In het darmepitheel van karperachtige vissen treedt reeds een half uur na orale toediening van antigenen een aspecifieke afweerreactie op.

Davina, J.H.M., et al., Develop. Comp. Immunol., 6 (suppl. 2): 157-166, 1982.

X

Veel landbouwkundig en medisch wetenschappelijk onderzoek kan worden omschreven als toegepast biologisch onderzoek.

XI

In fysiologie-leerboeken dient de huid als tastorgaan dat niet alleen informatie ontvangt maar ook verstrekt, minstens zoveel aandacht te krijgen als de zintuigorganen oog en oor.

Dijkhuis, Ned. Tijdschr. Fysiotherapie, 88: 34-41, 1978.

XII

In de fysiotherapie dienen de mogelijkheden van vroegtijdige onderkenning van een ontwikkelingsstoornis bij kinderen van 0-2 jaar en van de behandeling daarvan te worden verbeterd.

XIII

De bewaping is de kanker van onze maatschappij. (Dorothee Solle)

XIV

Zelfs in een land met een lange traditie van tolerantie moet de ruimte voor de ander en voor het anders zijn en denken door elke generatie opnieuw worden veroverd. (Koningin Beatrix)

XV

Verandering van structuren haalt niets uit, als de mentaliteit niet tevens verandert. (Geert Groote, 1340-1384)

XVI

Het inkrimpen van het universitair personeelsbestand zonder rekening te houden met de tijdelijk aangestelde onderzoekers met een up-to-date opleiding en ervaring is als het behandelen van infectieziekten zonder rekening te houden met het bestaan van bacterien en virussen.

XVII

Het vereenvoudigd grensoverschrijdend verkeer is een reden te meer om de stelling "He koômp oet Losser en weet van niks" te verwerpen.