The Reserve Cell in the Uterine Cervix

aspects of development, differentiation and diagnosis

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De reservecel in de cervix uteri detectie, differentiatie en diagnose.

Proefschrift

ter verkrijging van de graad van doctor aan
de Erasmus Universiteit Rotterdam op gezag van de
rector magnificus Prof. Dr. S.W.J. Lamberts en
volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
3 december 2008 om 15.45 uur

door

Johanna Elisabeth Martens
geboren te Haarlem

Promotiecommissie

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1

General introduction

Introduction

Carcinoma of the uterine cervix is worldwide the second most common cancer in women[1]. It has been approximately 150 years since the first description of uterine cervical carcinoma, a century since the description of its precursor lesions[2], and half a century since the introduction of the method proposed by Papanicolaou[3] for detecting cervical neoplasms by cytologic screening. In time investigators have proposed a multitude of histologic and cytologic terms for cervical precursor lesions[4;5], have devised methods and techniques for diagnosis and treatment[6;7], and have identified the causative agent i.e. human papillomavirus (HPV)[8].

Subclinical cervical HPV infection causes typical cytological and histological features called koilocytosis[9]. Koss was one of the first to relate koilocytosis to condylomas and associated these with premalignant cervical lesions, zur Hausen (Nobel Prize winner 2008) was the first to relate HPV to cervical cancer [10-12]. From that moment HPV was the major subject for investigators in the search for answers in cervical carcinogenesis[13]. In the 1980's and 1990's new molecular and cell biological techniques developed rapidly and made it possible to detect HPV DNA in tissue and cell samples[14], to identify the many HPV subtypes and to study the effect of HPV on the cell cycle and the process of apoptosis[15]. These techniques are now widely applied for diagnostic purposes, allowing a beter recognition of the HPV-infected lesions. Resulting in a better prognostication and giving rise to more molecular approaches that complement the (immuno) histochemical and cytochemical protocols.

HPV mediated carcinogenesis

The molecular model for HPV mediated carcinogenesis involves interaction of HPV gene products with the tightly controlled network of cellular oncogenes and tumor suppressor genes, which control cell proliferaton, apoptosis, DNA synthesis and DNA repair[16].

Persistence of a high risk HPV infection is a necessary condition in the carcinogenesis of cervical cancer. Integration of the viral DNA into the human genome is essential for progression[17]. The E6 and E7 gene products of the high risk HPV viruses, in particular HPV 16 and HPV 18, encode for proteins that interfere with the mitosis and apoptosis regulating pathways of the host cell, i.e. the p53 and the pRb signaling routes. Accordingly the E6/ E7 induced inactivation of these pathways results in hyperproliferation and inhibition of apoptosis, thus leading to genetic instability (amongst others numerical and structural chromosome aberrations) and immortalization[16-19].

HPV target cell

The link between morphological changes observed at colposcopy and microscopy on the one hand, and the causal role of HPV-induced genetic changes on the other, is still largely missing. The target cell in the uterine cervical epithelium undergoing the transforming mutations, caused by high risk HPV infection and incorporation in the cells genome, is still subject of speculation. The expression of the viral proteins is dependent on cell differentiation. It is suggested that when the integrity of the basal layer is compromised due to micro-traumata or environmental changes HPV can infect the progenitor/stem cell of cervical (pre)malignancy. The viral genome is maintained as low copy number episomes in the basal layer of the epithelium. Transcription of the viral genome is predominantly increased in the more suprabasal, differentiated layers. In light of carcinogenic models in comparable epithelia like esophageal, prostatic, colorectal and stomach epithelium, the putative target cell for HPV infection resulting in (pre) malignancy is most likely an epithelial stem cell of the healthy cervical epithelium occuring in the so called transformation zone [20-22].

Stem cells, a hierarchy of potential

Stem cells have varying potential[23], the most primitive, toti-potent stem cell is the fertilized oocyt, able to form the embryo and the trofoblast of the placenta. About 4 days after fertilization these toti-potent cells form a blastocyst and the inner cell mass from which the embryo develops. The cells of the inner cell mass are considered to be pluripotent, able to differentiate into almost all cells that arise from the three germ layers. Most adult tissues contain multipotential stem cells, e.g the hematopoietic stem cells, capable of producing a limited range of differentiated cell lineages appropriate to their location. At the end of this hierarchic tree the unipotential stem cells occur, capable of generating one specific cell type, such as for example epidermal stem cells. These unipotential stem cells are often called commited progenitors or progenitor cells. The subject of this thesis is to shed more light on the epithelial stem cells or progenitor cells of the human uterine cervical epithelium.

Since the normal and malignant cervical epithelium consist of at least two major differentiated cell types, i.e. the glandular cells and the squamous cells, one can raise the question whether these arise from one and the same stem cell compartment or whether two different stem cell types are needed for normal cervical epithelial genesis.

Stem cell concept in epithelial carcinogenesis

Pierce and Potten suggested that the target cell in the carcinogenic cascade is the stem cell[24;25]. In adult organisms each tissue and organ is generally accepted to contain a small subpopulation of cells capable of self renewal, of indefinite proliferative potential, and with the ability to give rise to a large family of descendants with defined spectra of specialization. A tissue specific stem cell or progenitor cell is normally multipotent, i.e. capable of producing a limited range of differentiated cell lineages. In normal circumstances tissue-specific stem cells generate the range of cell types appropriate to their location. In times of chronic damage or regeneration a process of metaplasia can give a switch in tissue differentiation. This metaplastic switch occurs at the level of progenitor cells rather than between terminally differentiated cells[23]. Examples include squamous metaplasia in airways of smokers, intestinal metaplasia in the stomach[26], and in the uterine cervix squamous metaplasia occurs frequently at the squamo-columnar junction[27]. It is likely that cancer,

particularly in continually renewing tissue, is in fact a disease of epithelial progenitor cells, since these are the only cells that persist in the tissue for a sufficient length of time to acquire the number of genetic changes needed for neoplastic development[23].

Arguments for stem cell based cervical carcinogenesis

Support for a stem cell origin of cervical carcinoma can be found in the fact that most cervical cancers originate in the so called transformation zone[28]. In this area the squamous metaplastic process takes place, i.e. reserve cell hyperplasia develops through immature squamous metaplasia to squamous epithelium[29]. This physiological regeneration process starting in the reserve cells, can be disturbed and than lead to the different stages of dysplasia and finally to invasive cervical carcinoma. Cell proliferation at the time of carcinogen exposure seems to be essential for fixation of any genotoxic injury into a heritable form, and therefore any cell in the cervical epithelium that has proliferative potential can be susceptible to neoplastic transformation[27].

Another important aspect of cervical carcinogenesis is the relationship between HPV and the stem cell for cervical carcinoma. The above mentioned model describes a stepwise influence of persisting HPV infection on the host cell genome, therefore it can be envisaged that the target cell must survive long enough to accumulate sufficient DNA instability for progress to invasive cancer. It is also known that it takes at least a decade to develop cervical carcinoma starting from the initial infection and low stage premalignant lesion. Since basal cells are the stem cells for epithelial regeneration, cervical cancer most likely originates from this cell compartment.

Invasive cancer develops from cervical intra-epithelial neoplasia (CIN), which develops in topographically defined fields. The transition from CIN to non-neoplastic fields is generally abrupt, without lateral spread or gradual transitions. Reich et al[30] propose therefore clonal proliferation of an individual stem cell may be responsible for repair of a distinct segment of the epithelium. Similarly, circumscribed fields of CIN may develop from such HPV-infected stem cells[30].

Stem cell candidates in the uterine cervix

Stem cells can be defined as undifferentiated cells, often present in the basal layer of an epithelium, with the capacity of self renewal and the ability to asymmetric division, thus producing committed daughter cells that can migrate into the suprabasal layers and differentiate.

The uterine cervix consists of an outer ectocervix and an inner endocervix. The ectocervix is lined by a multilayered squamous epithelium and the endocervix by glandular columnar epithelium, often with a subcolumnar layer of reserve cells. Three potential stem cell candidates can be distinguished in the uterine cervical epithelium, i.e. 1. the basal cell of the squamous epithelium, 2. the columnar cell and 3. the subcolumnar reserve cell. These three cell types are located at and interact with the basal membrane. As described above, the cervical stem cell however, should be multipotent.

The reserve cell can not only renew the columnar cell population but through the process of squamous metaplasia also the squamous cell population[27]. This makes the reserve cells (or at least a subpopulation of the reserve cells) serious candidates for a progenitor cell or even stem cell compartment.

The reserve cell

The reserve cell has been subject of investigation for over a century and its function and origin were intensely debated in the first half of the 20th century. In 1910 Meyer first, describes the reserve cell and the process of reserve cell hyperplasia[31]. He suggests that reserve cells originate at about six months of gestation when, because of the encroaching growth of columnar epithelium in the cervix, the pre-existing squamous epithelium is retracted, leaving basal-type squamous cells behind. Novak[32] suggested an ingrowth of the basal cells from the squamous epithelium at the squamo-columnar junction, undermining the columnar epithelium. Fluhmann[33] suggested that reserve cells originate from columnar cells by means of unequal division. Song[34] and Lawrence and Shingleton[35] indicated that reserve cells are derived from stromal cells. This was further supported by Reid et al[36], who suggested that stromal cells destined to be transformed into reserve cells are derived from

mononuclear cells of bone marrow origin. Recently Witkiewicz et al[37] concluded that reserve cells in microglandular hyperplasia can originate from specialized columnar cells.

The regenerative function of the reserve cell, as the cell from which the metaplastic process originates, was first described by Fluhmann[38]. Carmichael [39] initiated the concept of subcolumnar basal cells as reserve cell depots from which squamous and columnar cells could originate. This was largely based on the presence of mucin in the subcolumnar basal and metaplastic squamous cells. The multipotency of reserve cells was later confirmed by immunohistochemical studies based on specific detection of individual cytokeratin subtypes[40;41].

Epithelial stem cell markers

Tissue stem cells form the cellular base for organ homeostasis and repair. Stem cells have the unusual ability to renew themselves over the lifetime of the organ while producing daughter cells that differentiate into one or multiple lineages. Unlike hematopoietic stem cells, whose pluripotency can be verified in vivo using cell-transfer experiments, epithelial stem cells cannot readily be tested for functional competence. They are difficult to isolate and their proliferative capacity is strongly influenced by their environment, the so called stem cell niche[42]. Another problem is that epithelial stem cells are difficult to characterize because of the absence of specific molecular markers for each epithelium.

Candidate epithelial stem cell markers can be divided into several groups, including transcription factors, signaling proteins, detoxifying proteins, markers of immortality, etc. In the literature several stem cell markers have been used to detect these cells in the different types of epithelia, but not all of these markers give unequivocally conclusive results. For example, Oct 3/4 identifies pluripotent cells in human germ cell tumors[43], but studies on cervical carcinoma show no expression of this transcription factor[44]. Also stem cell abundant proteins like nanog, nucleostemin and musashi-1 are not expressed in normal uterine cervix[45]. Studies on Wnt-signaling pathways are limited to studies on mouse models where

several Wnt-signaling molecules have been shown to act together to establish the correct development of the uterus[46]. Furthermore, studies on catenins indicate a role for this family of proteins in the development and progression of neoplasm of the uterine cervix[47;48].

Another class of candidate stem cell markers is the integrin family of transmembrane receptors, whose members are responsible for the attachment of the basal cell layer to the basement membrane[49]. It is possible that stem cells require strong adherence to the basement membrane to maintain their stem cell characteristics or their position in the stem cell niche. Despite this, most, if not all proliferating cells use integrins in adhesion. Therefore the usefullness of integrins as stem cell markers is limited by the uncertainty of interpretation of their levels of expression relative to the transit-amplifying cells.

Immunohistochemical markers

For our studies we used paraffin material which enabled us to correlate morphology with immunohistochemical marker expression at single cell level. For the recognition of the different cell populations this type of fixed material is essential to allow correlation with aspects of development and differentiation of cell types. Only this type of material enabled us to perform the study as described in chapters 3, 4 and 5. Particularly the study on embryonic tissue, as described in chapter 2, would never have been possible without the use of paraffin material. A consequence of this approach is however the limitation that we could only apply antibodies that react in formalin fixed and paraffin embedded material. In our investigation of cervical carcinogenesis we focussed on the reserve cell. Our aim was to obtain more information on the characteristics, origin, phenotype, distribution pattern, and possible progenitor or stem cell features of these cells. Our immunohistochemical approach was based on newly proposed as well as previousy suggested stem cell markers and differentiation markers found in the developing human uterine cervix, in adult normal cervical epithelia and in its (pre)malign lesions.

We used the following immunohistochemical markers: bcl-2 plays a central role in the inhibition of apoptosis, and localizes to basal cells of the squamous epithelium and to subcolumnar reserve cells[50]. It was detected using an antibody to a synthetic peptid.

We hypothesize an epithelial stem cell must be protected against apoptosis to be able to survive as long as possible.

Ki-67 is a well known marker for proliferation and can be used in cervical epithelium[51]. Since progenitor cells are generally quiescent with a low proliferation capacity this marker should in general be negative in such cells[52].

P63, a homologue of the tumor suppressor p53, is a transcription factor operating mainly in the embryonal stage of development and plays a role in the regulation and maturation of the cervical epithelium in the adult phase[53-57]. A critical role for p63 in the normal development of the cervical epithelium was found in committings of early epithelial stem cells to a basal progenity[54]. In the absence of p63 basal progenitor cells (which are normally p63-positive) are absent and are replaced by ciliated columnar cells[58]. P63 is therefore important for the formation of progenitor cells at the basal layer of stratified squamous epithelium. Mice deficient of p63, completely lack stratified squamous epithelia as demonstrated by the lack of cytokeratin 14, a marker for commitment to squamous epithelia. The mice die soon after birth and display a number of developmental defects as well as several abnormalities in limb development[53]. P63 is particularly highly expressed in progenitor or stem cell populations of a variety of epithelial tissues[53;59-61].

Glutathione S-transferase π, a member of a multigene enzyme family that plays a role in the detoxification of endogenous and exogenous compounds, mainly catalyzing their conjugation with gluthatione[62]. It is found mainly in the cytoplasm and has been shown to have a detoxificating capacity for carcinogens [63;64]. Since stem cells by virtue of their role in tissue regeneration should possess efficient defense mechanisms for protection against DNA damaging agents, we expected this marker to occur in the stem cell of the uterine cervix. Shiratori et al.[64] have found positivity in koilocytotic cells and suggested that the enzyme expression could be related to the presence of HPV.

Cytokeratins (CK) were shown in earlier experiments to represent strong differentiation markers for different types of epithelial cells in the cervix [41]. Secondly, reports from the literature suggest that cytokeratins are suitable markers for progenitor or stem cells[65-67].

The cytokeratins are a family of intermediate filament proteins that are characterized by their molecular weight and in human epithelia numbered from 1 to 20. Cytokeratin expression occurs in cell-type specific combinations and can be used for identification and subclassification of epithelial tissues. In our study we used CK 5,14,17 and 19 for basal cell differentiation, CK 7,8 and 18 for glandular differentiation and CK 13 for squamous differentiation [41].

Aim of this thesis

The central theme of this investigation was a search for the progenitor/stem cell of the human uterine cervical epithelium. It is hypothesized that the reserve cell plays a crucial role in the regeneration of the different epithelial cell lineages of the uterine cervix, thus shedding more light on the missing link in cervical carcinogenesis, a progenitor cell for cervical cancer, the putative HPV-target cell.

Outline and scope

In chapter 2, fetal human tissue from different gestational ages was studied by using antibodies to p63, bcl-2, Ki-67 and cytokeratins 5,7,8,13,17,18 and 19, to ascertain when basal or stem cells first appear in cervical epithelium during fetal human development. It was presumed that relatively high concentrations of progenitor cells would be present during fetal human ontogenesis. This study also tried to solve the question of the origin of the reserve cell.

In chapter 3, an immunohistochemical study is performed using monoclonal antibodies against p63 and cytokeratin 17. A well defined subset of normal epithelium of the uterine cervix and in preneoplastic samples (CIN I, II and III) was used. By applying these markers for basal cells we tried to identify the progenitor cell of the uterine cervical epithelium.

In chapter 4, the distribution pattern and immunoprofile of reserve cells along the entire length of the adult cervix was examined by the use of p63, bcl-2 and cytokeratins 5,7,8 and 17 antibodies. Reserve cell subpopulations were studied with specific keratin phenotypes, being the progenitor cell population of the different types of the cervical epithelium.

In chapter 5, the detoxification enzyme Gluthation S-tranferase π (GST π) is used as a marker for progenitor cells to shed more light on the cervical carcinogenesis. In the uterine cervix the presence of GST π has been associated with high grade cervical intraepithelial neoplasia (CIN), but reports are conflicting. We investigated GST π expression immunohistochemically in a well documented sequence from normal epithelium to cervical cancer. In a search for the progenitor of the cervical epithelium, it was hypothesized that a potential stem cell must be protected against toxifing agents. Focus was placed on the expression of GST π in the basal epithelial cells and reserve cells.

Chapter 6 focusses on markers for progressive potential of premalignant lesions in cytologic smears, with particular interest in the identification of dysplastic cells in smears, using antibodies to cytokeratin 8 and cytokeratin 17. The use of these antibodies could be helpful in detecting premalignant lesions in cytologic smears or be informative of their progressive potential. Furthermore it was investigated whether reserve cells could be identified in cervical smears using these antibodies.

The General discussion, in chapter 7, provides an overview of our main results in relation to the literature on progenitor/stem cells in general and in carcinogenesis. We finally conclude that the reserve cell serves as the progenitor cell in uterine cervical epithelium, although functional studies still have to be performed.

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2

Reserve cells in human uterine cervical epithelium are derived from Mullerian epithelium at midgestational age.

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International Journal of Gynecological Pathology 2007; 26(4) 463-468

Abstract

Introduction

The role of endocervical reserve cells in squamous metaplasia and neoplasia is still debated. Their origin in the cervix is open to speculation and it is unclear, how they are targeted during carcinogenesis. In order to further understand the primary characteristics of reserve cells we phenotyped them in the developing human cervix.

Material and Methods

In 13 perinatal autopsies between 16 to 40 weeks of gestation, the human fetal cervix was evaluated in serial sections. Immunostaining comprized a panel of antibodies for cytokeratins, p63, bcl-2 and the sections were stained with alcian blue, as well as PAS before and after diastase treatment.

Results

Reserve cells are first identified at approximately 20 weeks of gestation. They are first noted under Müllerian type columnar cells lining the developing uterine cavity. There is a considerable overlap in expression profiles of Müllerian cells and reserve cells for p63, bcl-2 and cytokeratins 5, 8 and 18 at this stage of development, with increasing gestational age expression localizes to respective cell compartments . Eventually the phenotype of these cells correspond fully with that described for adult reserve cells and endocervical cells.

Conclusion

Müllerian epithelial cells are the stemcells for endocervical reserve cells and endocervical columnar cells. They have the capacity to transform into both endocervical columnar and squamous type epithelium in the endocervix, during early cervical development.

Introduction

Cervical cancer generally originates in the uterine cervical squamous columnar junction. This junction is lined by ectocervical squamous epithelium, endocervical columnar epithelium and basally located reserve cells. The reserve cell layer is able to regenerate the epithelium and is considered to harbour a stem cell population[1]. The process of reserve cell hyperplasia often progresses to squamous metaplasia resulting in the formation of squamous epithelium. Infection with high risk Human Papilloma Viruses (HPV) can disrupt this physiologic process resulting in transformation into a premalignant epithelial type[2]. Carcinogenetic theories speculate that epithelial stem cells are the target for HPV. Reserve cells therefore probably play a central role in the development of cervical cancer. Because little is known about these cells a comprehensive characterization could help in our understanding of cervical carcinogenesis.

Endocervical reserve cells in the adult cervix contain the p53 homologue p63, and most of these also cytokeratin 17[3].

At present there is no definite concept regarding the origin of these cells. A recent paper by Witkiewicz et al[4] on the evolution of reserve cells in microglandular hyperplasia concludes these cells can originate in the adult from specialized columnar cells. In contrast, other theories hypothesize that reserve cells are the progenitors from which columnar epithelial cells originate[1;5;6].

An early theory regarding the origin of these cells by Meyer in 1910, suggested that they originate at about 6 months of gestation, when due to encroaching growth of columnar epithelium in the cervix the pre-existing squamous epithelium is retracted, leaving basal type squamous cells behind[7].

Novak suggested an ingrowth of the basal cells from the squamous epithelium at the squamous columnar junction undermining the columnar epithelial layer[8]. Fluhmann was the first to conceive reserve cells to have a columnar cell origin, stating that they originate from columnar cells by unequal division[9]. Song and Lawrence and Singleton, believed that reserve cells derived from stromal

cells[10-12]. This was further supported by Reid who suggested that stromal cells are destined to be transformed into reserve cells are derived from mononuclear cells [13].

Using a totally different approach with molecular markers the group of Cunha suggests that squamous differentiation in the cervix is related to the initiation of p63 expression in the columnar type cells lining the Müllerian duct and part of the vagina[14].

To shed more light on the development of the different epithelial cell lineage in the cervix, we undertook a study in embryonic tissues attempting to identify the reserve cells as early as possible during human fetal development, as it can be expected that relatively high concentrations of reserve cells and their progenitors are present during early fetal human ontogenesis.

We therefore profiled the various cell populations in the developing fetal femaleurogenital tract using cytokeratin antibodies specific for luminal and basal cell compartments, in combination with the stem cell markers p63 and bcl-2.

Material and methods

Specimens and processing

From the files of the Department of Pathology of the Foundation of Collaborating Hospitals in Eastern Groningen we selected 13 perinatal autopsies of gestational ages between 16 to 40 weeks. Fetal age was determined using foot length tables and femur X rays. Cases were selected, if 1. there was an evaluable junction between epithelial lining of the early uterine corpus and the most cranial portion of the vagina, 2. autolysis was not too extensive, 3. there was enough tissue available for further evaluation in semiserial sections, and 4. there was no evidence of congenital malformations.

Table 1. Antibodies and antigens used in this study with retrieval procedures applied.

Antibody/ antigen/ Cytokeratin Detected	Antibody clone	Antigen retrieval step/pH	Source
P63	4A4 +, Ab4 Y4A3	EDTA 8,0	Neomarkers, Fremont, California USA
Ki-67	7B11	EDTA 8,0	Zymed, San Francisco, USA
Bcl- 2	124	Tris/EDTA 9,0	Dako A/S, Glostrup, Denmark
CK5	XM-26	Tris/EDTA 9,0	Novocastra, Newcastle upon Tyne, UK
CK7	OV-TL 12/30	protease	BioGenex, San Ramon, California SA, USA
CK8	CAM 5.2	protease	Becton Dickinson, USA
CK13	1C7	Citr 6,0	Neomarkers, Fremont, California, USA
CK17	E3	Citr 6,0	Neomarkers, Fremont, California USA
CK18	RCK106	Citr 6,0	MUbio Products BV, Maastricht, The Netherlands
CK19	RCK108	protease	MUbio Products BV, Maastricht, The Netherlands

Results were catagorized into three gestational age groups i.e. from 16 to18 weeks (n=2), from 19 to 24 weeks (n=6), and from 29 to 40 weeks (n=5). We also took paraffin sections from adult cervix as controls (n= 2).

Serial sections were cut from the paraffin blocks and subjected to immunostaining with a panel of antibodies (Table 1). We also stained sections for H&E, Alcian blue and PAS before and after diastase treatment. The Medical Ethical Committee of the Foundation of Collaborating Hospitals of Eastern Groningen approved this study.

Slide evaluation

In each case we evaluated the columnar type epithelium lining the fused Müllerian ducts. In those cases in which there was an evident junction between the squamous type epithelium lining of the cranial part of the primitive vagina and columnar epithelium we particularly investigated this area. The immunoreactivity of the various types of epithelium was semiquantitatively scored. Immunostaining was scored as weak, moderate or strong in comparison to expression in the adult cervix.

The staining results were independently evaluated by four of the authors (JM, FS, RvM and JWA). In cases of discrepancy, slides were reviewed together and consensus was reached in all cases.

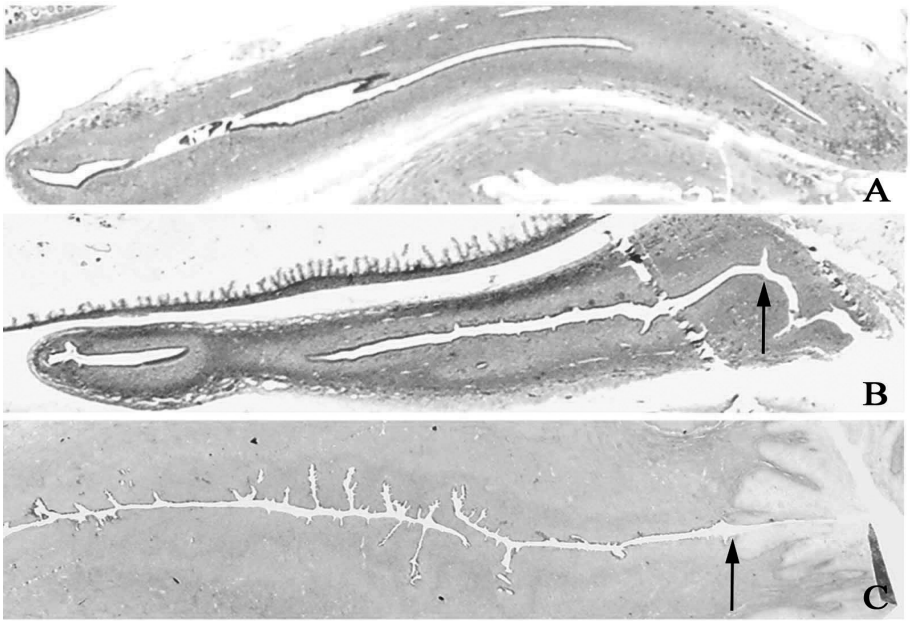
Results

Histological features of cervix development

In order to identify the reserve cell population in the fetal cervix, we focussed on the Müllerian epithelium particularly in the proximal portion of the early uterine cavity.

Figure 1 shows an overview of the uterine cavity of three different gestational stages. Figure 2 summarises the development of the immunohistochemical profile of the columnar epithelium and reserve cells in the developing human uterine cervix.

Figure 1 (figure in color on page 141)

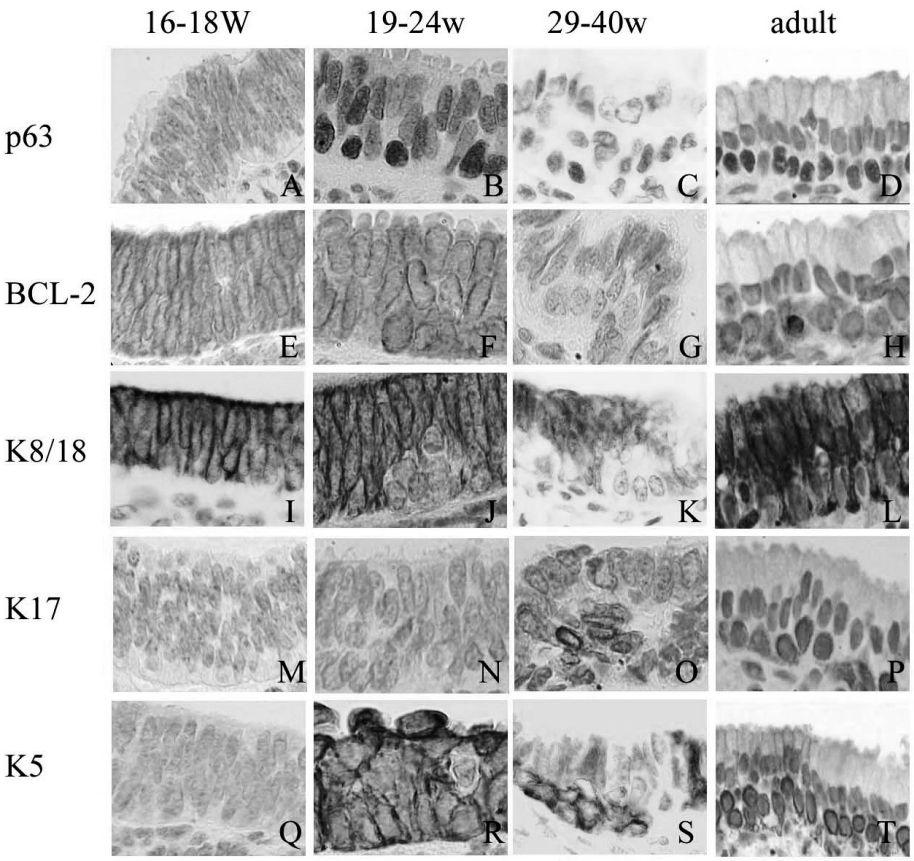


a.) Overview of the lower genital tract in a fetus gestational age 18 weeks. The primitive uterine cavity is present (left) and there is a solid cord of epithelial cells in the distal portion of the uterine cavity (right, Mullerian ducts). The transition between the uterine cavity and solid epithelial cord has yet to be established.

b.) Overview of lower genital tract in a 19 week old fetus. The transition between uterine cavity and solid epithelial cord has been established (arrow) The observed lumen is an artefact due to retraction of the solid epithelial cord from the underlying stroma. In the proximal portion of the primitive uterine cavity there is no endocervical differentiation.

c.) Overview of the lower genital tract in a fetus gestational age 29 weeks. The arrow denotes the transition between the ectocervix, lined by non-keratinising squamous epithelium and the newly developed endocervix. This is very short (less than 1mm) and lined by endocervical and Mullerian type columnar cells.

Figure 2 (figure in color on page 142)



The development of reserve cells in the human fetal uterine cervix from 16-18 weeks, 19-24 weeks, 29-40 weeks of gestation to adult and the development of the expression pattern of p63, bcl-2, cytokeratins 8,18,17 and 5 in the reserve cells and the overlying columnar epithelium.

In the fetuses of 16 and 18 weeks gestation there was no discernable junction between squamous epithelium lining of the vagina and the proximal portion of the uterine cavity which had not developed any cervical characteristics. The distal uterine cavity was funnel shaped and was lined by columnar cells with large nuclei and little cytoplasm (figure 1 A).

In the fetuses between 19 and 24 weeks the funnel shaped uterine cavity first abutted a solid epithelial cord, this could be considered an early squamocolumnar junction (figure 1 B). Reserve cells were first noted at approximately 20 weeks of gestation. They were identified under the Müllerian type epithelial cells, often also not directly adjacent to the early transitional zone (figure 2 B). In a few cases, in which there was as yet no discernable contact between Müllerian epithelium lining the primitive uterine cavity and the more distally located solid squamous epithelial cord, reserve cells could also be identified under the Müllerian epithelial cells.

At 29 weeks gestation a squamocolumnar junction showing adult features was first recognized (figure 1 C). This consisted of the appearence of small numbers of columnar cells with containing mucins. Furthermore as opposed to the Müllerian cells these cells showed small nuclei basally located. The solid squamous like epithelial cord had split open and there was an abrupt transition between the squamous epithelium and columnar epithelium. (figure 2 C). Underlying basal cells were observed reminescent of the reserve cell population in the adult squamocolumnar junction.

At 29 to 40 weeks there were still a considerable number of Müllerian type cells in the transitional zone but many had transformed into slender columnar type cells with small basally located nuclei and abundant cytoplasm containing acid mucopolysacharines. Under both columnar cell types (Müllerian and endocervical), small numbers of reserve cells could be distinguished in all cases.

Immunophenotyping studies

In the 16 and 18 week gestation fetuses (n=2) there was no discernable junction between the two different types of epithelium lining the lower genital tract. The uterine cavity was lined by Müllerian type columnar cells. Upon close inspection basal cells under the columnar cells were not identified. The columnar cells intensely expressed the luminal type cytokeratins 8 and 18 (figure 2 I) and bcl-2 (figure 2 E) The basal cell marker cytokeratin 5 was present in a minority of cells (figure 2 Q). Cytokeratin 17 and p63 were not detected at this stage (figure 2 A and figure 2 M).

The 19 to 24 week gestational age fetuses (n=6) showed a change in the immunostaining compared to the younger fetuses. In these cases we were able to identify reserve cells in the distal part of the uterine cavity. P63 and bcl-2 were intensely expressed in the reserve cells, the overlying columnar cells also expressed these markers less intensely (figure 2 B and figure 2 F) . The level of cytokeratin expression of the reserve cells was different from the overlying columnar cells which helped in their identification. Of the simple cytokeratins, cytokeratin 7 was intensely expressed, while cytokeratins 8 and18 were less intensely expressed than in the overlying columnar cells (figure 2 J). Basal type cytokeratin 5 (figure 2 R) was intensely expressed along with cytokeratin 13. Cytokeratin 17 could not be detected in either compartment (figure 2 N).

In the fetuses of 29-40 weeks gestation (n=5) there was a recognizable squamocolumnar junction with the appearence of small numbers of endocervical cells. P63 stained the reserve cells, while overlying endocervical type columnar cells were negative (figure 2 C). However Müllerian type columnar cells were still weakly positive. Furthermore there was staining activity for bcl-2 in most reserve cells (figure 2 G). Cytokeratin 8 and 18 showed strong expression in the Müllerian epithelium and moderate immunoreactivity in the reserve cells as well as in the overlying columnar epithelium (figure 2 K).

Interestingly the reserve cells underlying the endocervical columnar cells from 29 weeks on showed a different cytokeratin expression pattern compared to the basal cells in 19 to 24 week gestational age fetuses. This was characterized by the additional expression of cytokeratin 17 along with more intense expression of cytokeratin 5 (figure 2 O and figure 2 S), indicating maturation towards an adult reserve cell cytokeratin profile.

In the adult squamocolumnar junction the reserve cells are strongly positive for p63 while the overlying columnar epithelial cells are negative (figure 2 D). The reserve cell population is also bcl-2 positive (figure 2 H), cytokeratin 5 and 17 positive (figure 2 T and figure 2 P). Cytokeratin 8 and 18 show strong positivity for the overlying columnar cells and lower activity in reserve cells (figure 2 L).

Discussion

The immunophenotype of reserve cells

The human uterine cervix is lined by ectocervical squamous epithelium, endocervical columnar epithelium and a subcolumnar compartment of reserve cells.

In the adult cervix reserve cells demonstrate a specific phenotype, expressing amongst others bcl-2 and p63 as well as cytokeratin 17 [3;15;16]. The main issues addressed in this study are 1. what is the origin of reserve cells and 2. in which way are reserve cells related to columnar and squamous cells? To answer these questions we focussed on the development of reserve cells in the fetal uterine tract.

This study shows that in the earliest stages of development of the uterine tract up to 19 weeks, reserve cells are not discernable. At about 20 weeks gestation a small number of basal cells (reserve cells) are noted underlying the columnar Müllerian type cells. Initially, these cells have an identical immuno phenotype as compared to the overlying cells, in terms of expression of p63, cytokeratin 5 and bcl-2, but the intensity of the expression is higher in the reserve cells.

We postulate that the Müllerian columnar cells are the progenitors of these reserve cells.

As gestational age increases, p63 and cytokeratin 5 expression in the overlying Müllerian type columnar cells decreases in comparison to the reserve cells. At approximately 29 weeks gestation a portion of the reserve cells initiate expression of cytokeratin 17, in distinction to the overlying columnar cells. Also in our previous study on cytokeratin 17 in the adult cervical epithelium cytokeratin 17 was identified as a specific marker for reserve cells [3].

Origin of reserve cells

According to the literature there are several theories and questions regarding the origin of reserve cells. In 1910, Meyer published data suggesting that reserve cells are remnants of fetal squamous epithelium which lined the uterine tract at an earlier phase of development [7]. Our study provides no proof for this theory as the part of the Müllerian tubes we investigated is lined by columnar epithelium during all phases of development and reserve cells appear beneath these cells at approximately 20 weeks.

Song suggested that these reserve cells are derived from subepithelial stromal cells [12]. Our study does not support this theory, as stromal cells do not express p63, or cytokeratins which has also been confirmed in adult studies [17].

Recently Witkiewicz et al published a study on microglandular hyperplasia suggesting that reserve cells are created in adulthood through a transition from columnar noted p63 expression in mature columnar cells (4) and the cytokeratin profile of these two cell types are different. Of course we cannot rule out the possibility that during very explicite conditions i.e. microglandular hyperplasia endocervical cells have the capacity to initiate expression of p63, and switch their cytokeratin pattern. Alternatively, one could speculate that the endocervical compartment is not homogeneous, consisting not only of mature endocervical cells but also containing small numbers of pluripotent Müllerian type cells that can dedifferentiate into reserve cells.

Our study indeed shows that Müllerian epithelium, transiently expresses p63 in early developmental stages and during this period it is capable of generating reserve cells. Transplantation experiments, in which urogenital sinus lined by Müllerian columnar cells of embryonal mice is grafted onto adult mice, show that reserve cells develop from Müllerian columnar type cells in the complete absence of squamous type cells, further supporting our observation, that Müllerian type cells are progenitors for reserve cells [18].

In a mouse and human embryo study the group of Cunha [14] looked closely at the development of squamous type epithelium in the female genital tract, amongst others by investigating the expression of different isoforms of p63. They noted that p63 can be

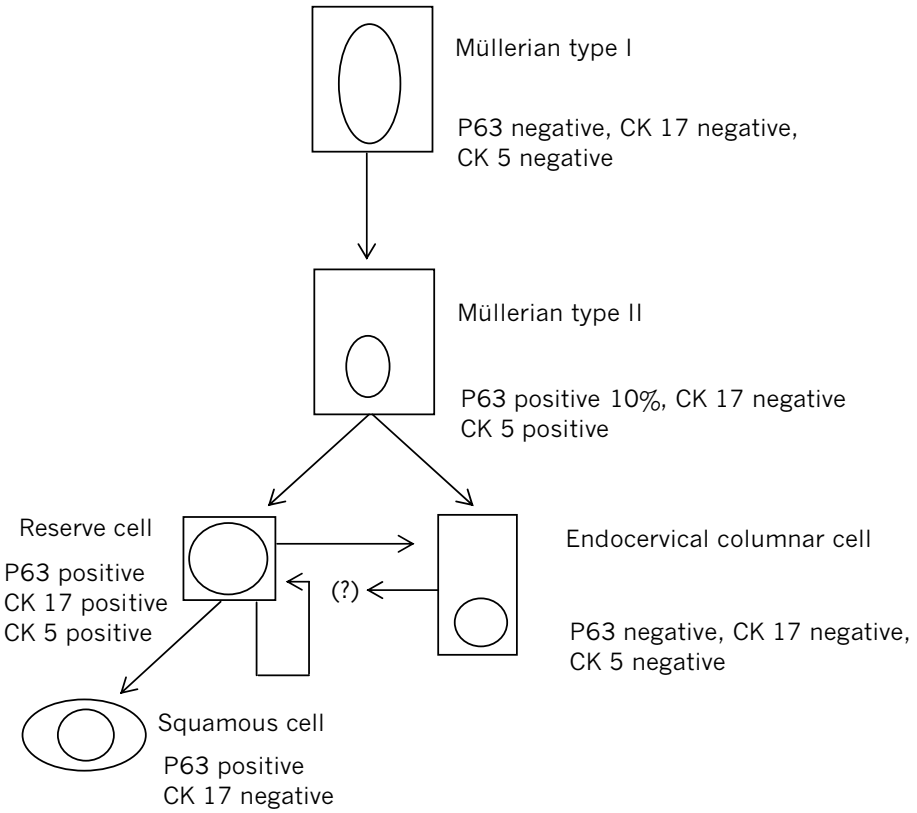
expressed in Müllerian type epithelial cells, confirming our study. These authors suggest that the expression of p63 imports a differentiation characteristic to this epithelium, which enables it to transform into a squamous type epithelium. Our observations enabled us to add to these observations by demonstrating that this transient expression enables the development of a basal type cell, the reserve cell. This newly appeared cell-type, is most likely the pool from which squamous metaplastic epithelium develops in later life. Although, as Witkiewicz points out, this may not be the only mechanism for squamous metaplasia (4).

Model for epithelial development in the human cervix

The Müllerian ducts are lined by columnar epithelial cells, up to the point where this epithelium abuts the solid epithelial cord that develops from the urogenital sinus. Up to about 20 weeks of gestation the Müllerian cells have limited differentation characteristics. At approximately 20 weeks a population of cells under the overlying Müllerian columnar cells arise. These cells contain the same markers as the Müllerian type cells, but p63 and cytokeratin 5 expression are more extensive and more intense. We postulate these cells to be the cell from which both reserve cells and columnar cells originate (figure 3). As gestational age increases a subpopulation of reserve cells, expressing cytokeratin 17, can be identified. We suggest this to be a reserve cell population, which has also the capacity to differentiate into a squamous direction.

In summary, our study shows that developing Müllerian epithelium generates the reserve cell population in the human cervix. Probably there are subpopulations of reserve cells giving rise to columnar or squamous epithelium.

Figure 3



Model for hierarchical cell lineage in the human uterine cervix. Early columnar type I (Müllerian) cells lining the Müllerian tubes express only simple keratins and are p63 negative. At approximately 20 weeks gestation expression of p63 and the basal cell marker CK5 is initiated in a small fraction of these cells, we call these cells columnar cell type II (Müllerian) cells. Simultanously we note the appearence of the first reserve cells under these type II columnar cells. Initially the p63 and keratin phenotype of both cells is identical. However, with increasing gestational age the reserve type cells aditionally express keratin 17. At approximately this time true endocervical cells appear. These cells arize either directly from columnar type II (Müllerian) cells during which process p63 and K5 are lost or from reserve cells. Whether endocervical cells give rise to reserve cells directly, has in our opinion not been proven satisfactorily to date. Reserve cells sustain their own numbers by low frequency mitoses and are of course the progenitor cells for squamous epithelium.

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3

Cytokeratin 17 and p63 are markers of the progenitor cell of the human uterine cervical epithelium, a putative HPV target cell.

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Based on:
Anticancer Research 2004; (24) 771-776

Abstract

Background

Basic research on HPV has focused on identifying the genetic changes that lead to cervical carcinoma. However, while focusing on the molecular biology of the cancer, understanding of its cellular biology has lagged: the target cell of the HPV infection is unknown.

Materials and Methods

In this study we identified the stem cell population of the cervical epithelium by monoclonal antibodies against p63, a homologue of the tumor suppressor gene p53 and cytokeratin 17 (CK17).

Results

We noted p63 expression consistently in the nuclei of reserve cells, hyperplasia of the reserve cells and the basal layer of the ectocervical epithelium, while CK17 only stained endocervical reserve cells and reserve cell hyperplasia.

Conclusion

We conclude that both p63 and CK 17 are suitable markers for cervical progenitor/stem cell identification. Both markers, therefore, qualify for the identification of the putative HPV target cell.

Introduction

The relationship between the development of cervical cancer and infection with certain types of Human Papilloma Viruses (high risk HPV) is well established[1]. Cell cycle is influenced by molecular interactions of human papillomavirus gene products, particularly from the E6 and E7 open reading frames. These gene products have the ability to bind host regulatory proteins and lead to degradation of the p53 tumor suppressor gene product E6 and functional inactivation of the (tumor suppressor) retinoblastoma gene protein (pRb) E7 [2;3].

However, the target cell of these transforming mutations, caused by high risk HPV infection in the uterine cervical epithelium still remains unknown.

Pierce and Potten suggested that the target cell in the carcinogenic cascade is the stem cell of the epithelium [4-6]. Stem cells are defined as cells that have the ability to perpetuate themselves by means of self renewal and to generate mature cells of a particular tissue through differentiation. Signalling pathways that normally regulate stem cell self renewal can lead to carcinogenesis when dysregulated, so stem cells may be the target of transformation. Arguments supporting this hypothesis are the following: firstly, stem cells have the machinery for self renewal already activated; secondly, stem cells often persist for long periods of time, in contrast to dying after short periods of time like mature cells in highly proliferative tissues. This means that there is a much greater opportunity for mutations to accumulate in individual stem cells than in mature cell types [7].

Therefore, it may be presumed that the target cell for high risk HPV infection is the stem cell of the uterine cervical epithelium. However, the exact nature of this stem cell is still under debate.

The uterine cervical epithelium consists of ectocervical squamous epithelium, endocervical columnar epithelium and subcolumnar reserve cells, so there are several potential candidates to qualify as stem cells. From morphological studies we know that reserve cells are undifferentiated, omnipotent cells which possess the capacity to undergo squamous differentiation (metaplasia) [8]. Basal cells of the ectocervical squamous epithelium

are more differentiated, dedicated to the formation of squamous cells, and therefore less suitable as stem cells of the epithelium [9;10].

Another problem in the identification of the stem cell is that, so far, reliable markers are not available to identify stem cells of certain epithelia. From earlier studies we noticed two potential markers [10;11].

Recently, p63, a homologue of the tumor suppressor gene p53, has been described as a transcription factor operating mainly in the embryonal stage of development [12]. In several tissues such as bronchial, prostate and cervical reserve cells, p63 has been immunohistochemically demonstrated and hence is suggested to play a role in the regulation and maturation of epithelium in the adult phase [11;13-15]. In pulmonary epithelium, p63 expression was seen in the bronchial reserve cells, which is consistent with the role of p63 in maintaining a stem cell population [16]. In prostate tissue, p63 specifically labelled basal cells [17]. P63 therefore could be a suitable marker for cervical stem cells [13].

Keratin polypeptide patterns can be used for identification or at least sub-classification of epithelial tissues. The keratin expression pattern in cervical tissue has been well defined [18]. Previously, we observed that subcolumnar reserve cells, a potential stem cell population of the uterine cervix, showed a typical keratin expression pattern of which cytokeratin 17 was prominent and specific [19]. To identify the stem cell of the uterine cervical epithelium, we performed an immunohistochemical study using monoclonal antibodies against p63 and CK 17 in a well defined subset of normal epithelium of the uterine cervix and in preneoplastic samples (CIN I, II and III).

Materials and Methods

Tissue specimens

All of the formalin-fixed and paraffin-embedded uterine cervix specimens were retrieved from the files of the Department of Clinical Pathology, Deventer Hospital, The Netherlands. Biopsies and diathermy loop excision specimens were taken from women with

cytologically verified dysplasia. Histopathological analyses were performed on H&E-stained sections. The samples comprised CIN I (6 cases), CIN II (7 cases) and CIN III (7 cases). In these samples normal ectocervical squamous epithelium was diagnosed in 14 cases: endocervical columnar cells (20 cases), reserve cells (17 cases) and reserve cell hyperplasia (5 cases) were also identified.

Immunostaining protocol

The p63 mouse monoclonal antibody (Ab 4, clones 4A4+Y4A3, titre 1:100) was used to study tissue samples. It was obtained from Neomarkers, Klinipath, NL. To perform the immunological staining procedures we used the Ventana Medical Systems iViewTM DAB Detection Kit, an indirect biotin streptavidin system of the Ventana BenchmarkTM (Ventana Medical Systems, Inc., Tucson, Arizona, USA). The specificity of the staining with p63 antigen was verified by an internal control system. In each case, stromal cells, that did not show p63 expression in animal models, were used as an internal negative control [11].

Mouse anti-cytokeratin 17 (clone E3) titre 1:200 was used to study the paraffin-embedded tissue samples in the same way, also using the Ventana BenchmarkTM and the Ventana iViewTM DAB Detection Kit. This antibody labels basal and myoepithelial cells of complex human epithelia [20]. The mouse anti-cytokeratin 17 antibody was obtained from Dakocytomation (Glostrup, Denmark).

Immunoenzyme double-staining method

We used an immunoenzyme double-staining method for the simultaneous detection of cytokeratin 17 and p63 antigens. The first indirect method involved an unlabelled monoclonal antibody cytokeratin 17 followed by the Ventana Medical Systems iViewTM DAB Detection Kit. After a washing step with Tris-HCl buffer pH 7.6, the second indirect method was applied using an unlabelled antibody p63 followed by the visualisation of this antibody by the alkaline phosphatase conjugated EnvisionTM reagents, based on a unique enzyme conjugated polymer backbone (Dakocytomation). The two antigens can be distinguished clearly and selectively by the reaction products of the enzyme activities of horseradish peroxidase (brown) and alkaline phosphatase (blue).

Evaluation of the staining reactions

The staining results of p63 and CK17 were independently evaluated by three of the authors (JM, BB and JWA). In cases of discrepancy, slides were reviewed together and consensus was reached in all cases.

The number of cells positively stained for p63 and CK 17 was semi-quantitatively evaluated. Four groups could be distinguished, i.e. cases with 1 to 25%, 26-50%, 51-75% and 76-100% of positive cells, respectively. For both p63 (nuclear staining reaction) and CK 17 (cytoplasmatic staining reaction), the expression within the different epithelial layers was studied. CIN lesions were subdivided into three compartments, i.e. basal, intermediate and superficial, each comprising, approximately one-third of the epithelial thickness. Staining in ectocervical squamous epithelium, endocervical columnar epithelium and reserve cells as well as reserve cell hyperplasia was separately identified.

Results

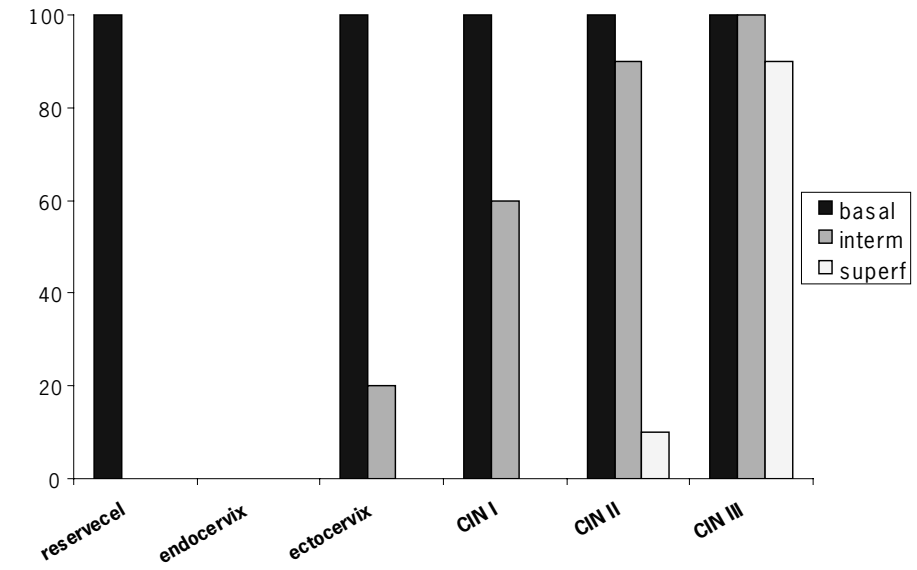
The immunohistochemical staining pattern of monoclonal antibody against p63 is schematically represented in Figure 1 and illustrated in Figure 3 A-F. The expression of the monoclonal antibody against CK 17 is represented in Figure 2 and further illustrated in Figure 3G-H. The results of the combined immunohistochemical staining of p63 (nuclear) and CK 17 (cytoplasmatic) staining illustrated in Figure 3 I.

Normal cervical epithelia

P63 expression was restricted to the nucleus and was consistently expressed in all slides. Staining intensity varied very little between individual cases. In ectocervical squamous epithelium, nuclear p63 staining was restricted to the basal layer of the epithelium in 76% to 100% of the cells in all cases (Figure 3A). In sharp contrast, endocervical columnar epithelium did not show any expression of p63. The subcolumnar reserve cells in the transitional zone and endocervix, however, showed p63 expression in 76%-100% of cells in all cases (Figure 3B). Clusters of reserve cell hyperplasia displayed intense nuclear immunoreactivity in all cases, in 76%-100% of cells (Figure 3C). In regions with koilocytotic changes p63 expression was completely absent.

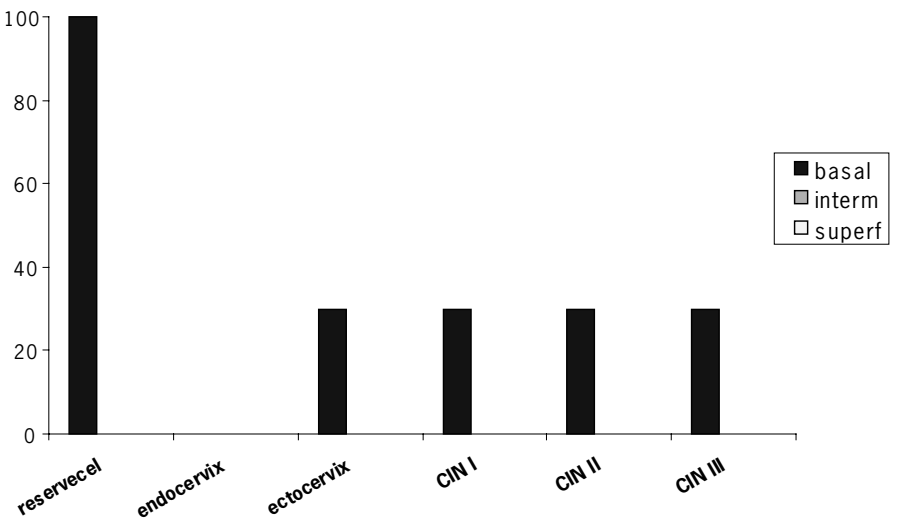
CK 17 expression was restricted to the cytoplasm and was consistently expressed in all slides. In ectocervical epithelium, CK 17 expression was noted in the basal compartment in 26-50% of cells in all cases. In endocervical epithelium, CK 17 was not expressed (Figure 2). The subcolumnar reserve cells in the transitional zone and in the endocervix, however, showed CK 17 expression in 76%-100% of cells in all cases (Figure 3G–H). Since p63 expression is nuclear and CK 17 expression is cytoplasmic, combined staining was possible and was demonstrated in 76%- 100% of cells in all cases (Figure 3I).

Figure 1



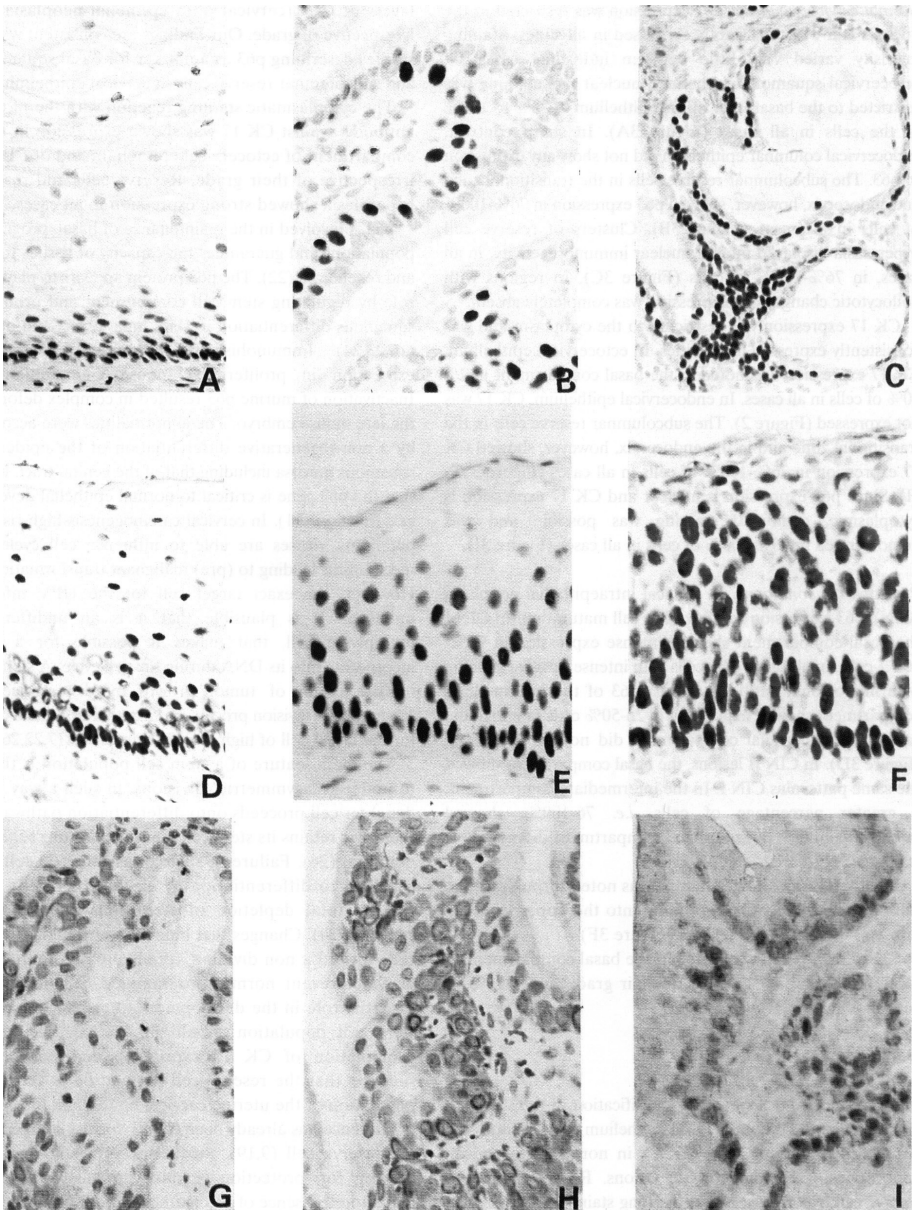
P63 immunostaining pattern in normal ectocervical epithelium, endocervical epithelium, reserve cells and CIN I-II-III lesions.

Figure 2



The CK 17 staining pattern in normal ectocervical, endocervical epithelium, reserve cells and CIN I-II-III lesions.

Figure 3 (figure in color on page 143)



P63 expression in normal ectocervical epithelium (A), endocervical epithelium and reserve cell (B), reserve cell hyperplasia (C), in CIN I (D), CIN II (E), CIN III (F). Cytokeratin 17 expression in endocervical epithelium and reserve cells (G), reserve cell hyperplasia (H) and double staining of p63 and CK 17 (I).

Preneoplastic conditions

In cervical intraepithelial neoplasia (CIN), p63 expression was linked to cell maturation. In CIN I the basal compartment showed intense expression in 76%-100% cells, in all cases. The cells with intense positive staining with monoclonal antibody against p63 of the intermediate compartment showed expression in 26-50% cells in individual cases. The superficial compartment did not show staining (Figure 3D). In CIN II lesions, the basal compartment showed the same pattern as CIN I. In the intermediate compartment, a greater percentage of cells, i.e. 76-100%, showed immunostaining. The superficial compartment did not show staining (Figure 3E).

In CIN III lesions, the staining was noted throughout the full epithelial thickness, extending into the upper layers in less well-differentiated lesions (Figure 3F).CK 17 expression was noted in the basal compartment of all CIN lesions, irrespective of their grade, in 26-50% of cells in all cases (Figure 2).

Discussion

In this report we described the identification of the stem cell population of the uterine cervical epithelium using monoclonal antibodies against p63 and CK 17 in normal ectocervical, endocervical and preneoplastic lesions. Reserve cells and reserve cell hyperplasia showed strong staining expression of p63 (nuclear) and CK 17 (cytoplasmatic) in all cases.

In all cases p63 immunostaining was strongly expressed in the basal layer of ectocervical epithelia and in the basal layers of CIN (cervical intra epithelial neoplasia) lesions irrespective of grade. Our findings are consistent with recent studies describing p63 as a marker for basal squamous cells and subcolumnar reserve cells in cervical epithelium [13;21].

The cytoplasmatic staining reaction with the monoclonal antibody against CK 17 was shown expression in the basal compartment of ectocervical epithelium and of CIN lesions irrespective of their grade. Reserve cells and reserve cell hyperplasia showed strong expression in all cases.

P63 is involved in the maintenance of basal, progenitor cell populations and guarantees the capacity of tissues to develop and regenerate [22]. The p63 protein appears to play a critical role by regulating stem cell commitment and promotion of squamous differentiation in skin, lung, cervix and other sites [16;23;24]. Immunohistochemical studies detected p63 expression in proliferating mouse and human tissues. Inactivation of murine p63 resulted in complex deformities in the late mouse embryo. The abnormalities were accompanied by a non-regenerative differentiation of the epidermis and squamous mucosa including that of the genital tract, indicating that the p63 gene is critical to normal epithelial development and function [11]. In cervical carcinogenesis high-risk human papilloma viruses are able to influence cell cycle control mechanisms leading to (pre) malignant transformation [1;25]. However, the exact target cell for the HPV infection is unknown. It is plausible that it is an undifferentiated, multiplying cell, that makes it possible for a virus to incorporate into its DNA during the cell cycle. A stem cell can be the origin of tumor growth by disturbance of

the "asymmetric" division process and can therefore be a candidate for the target cell of high risk HPV infection [7;22;26].

The main feature of a stem cell population is the ability to undergo "asymmetric" divisions, in such way that one daughter cell proceeds onto differentiation pathways, while the other retains its stem cell identity and thus regenerative potential [26]. Failure of such an asymmetric cell division can lead to differentiation of all daughter cells, thereby causing total depletion of stem cells and regenerative capacity [24]. Changes that block the normal maturation of cells toward a non dividing, terminally differentiated state or that prevent normal programmed cell death play an essential role in the development of cancer. Such a basal, stem cell population should be p63-positive [23]. The combination of CK 17 expression and p63 expression suggests that the reserve cell population is the stem cell population of the uterine cervical epithelium.

Evidence has already been found for the stem cell role of the reserve cell [9;19]. Furthermore, a study on bcl-2, a marker for protection against apoptosis, showed the ubiquitous presence of this marker in reserve cells [27]. This protection of bcl-2-positive reserve cells against apoptotic cell death is required to ensure survival of the epithelium and in this way points out their stem cell function.

The well-known process of metaplasia in the transformation zone of the cervix also suggests an important role for the reserve cell as the basal cell for columnar as well as squamous epithelial regeneration [8]. In our study we detected two important p63- and CK 17- positive cell populations, i.e. the basal cells of the normal ectocervical epithelium and the subcolumnar reserve cells. In conclusion, our study identified the reserve cell as the most favourable candidate for progenitor cell of the epithelium of the uterine cervix and showed the value of both CK 17 and p63 as markers for these stem cells.

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4

Distribution pattern and markerprofile disclose two subpopulations of reserve cells in the endocervical canal.

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Submitted

Abstract

Introduction

Our earlier study on the fetal uterine cervix provided evidence for the existence of two subpopulations of reserve cells [1]. Therefore reserve cells in the adult uterine cervix were investigated for their distribution pattern and marker profile.

Materials and Methods

Sagital sections from ten normal uteri, comprising the region from ectocervix to lower uterine cavity, were histologically examined and immunostained for p63, bcl-2 and cytokeratins (CKs) 5,7,8 and 17.

Results

The endocervical canal consists of three regions, i.e a part lined by squamous epithelium, a part lined by endocervical cells and a part lined by tubal type epithelial cells. Histologically we found reserve cells in all 10 investigated cervices, with a higher abundance under the endocervical columnar cells close to the squamous columnar junction, and high in the endocervical canal where the invaginations exist of tubal type epithelium. In between an area lined with endocervical columnar cells without reserve cells was identified. No reserve cells were detected in the endometrial epithelium. We defined the end of the endocervix as the point were the surface of the cervical canal and the invaginations are completely lined by tubal type epithelium. From this point upwards reserve cells are no longer found.

Reserve cells show strong expression for p63 and CK 5, CK 7 and moderate expression for bcl-2. CK 17 is strongly expressed in the area of the squamocolumnar junction and decreases in proximal direction. Endocervical columnar cells usually express CKs 7 and 8, sporadically also p63and CK 5. CK 17 was only found in these cells in the vicinity of CK 17 positive subcolumnar reserve cells. Tubal type epithelium was present in all samples and contains bcl-2, along with CKs 5, 7 and 8. Bcl-2 and CK 5 expression distinguishes tubal epithelium from endocervical columnar cells.

Conclusion

We conclude that reserve cells are present in all investigated cervices along the entire cervical canal. The concentration of reserve cells is highest proximal to the squamous columnar junction and in the upper third of the cervix. The marker profile of reserve cells is the same in all parts of the cervix exept for CK 17 which shows a decreasing gradient from distal to proximal. This suggests a subpopulation of CK 17 negative reserve cells proximal in the endocervical canal that can be the progenitor cells of columnar epithelium only.

Introduction

Premalignant lesions of the uterine cervix and subsequent cervical carcinoma generally originate in the uterine cervical squamo-columnar junction [2,3], which is formed at the interface between mature and/or immature squamous epithelium and endocervical columnar epithelium. The original squamo-columnar junction i.e the point where native ectocervical squamous columnar epithelium abuts endocervical columnar epithelium is often replaced by metaplastic epithelium during reproductive years. The new or functional squamo-columnar junction than becomes the region of active replacement of columnar endocervical epithelium by squamous cells. The area between the old and new squamocolumnar junction is the so-called transition zone. The endocervical canal is lined by a layer of columnar epithelium consisting of mucin secreting cells, between which ciliated cells, goblet cells, and usually dispersed subcolumnar reserve cells are found. It is suggested that from these reserve cells squamous metaplastic epithelium can originate, which then replaces the columnar epithelium. Reserve cell hyperplasia usually progresses to immature squamous metaplastic epithelium, resulting in the formation of mature squamous epithelium [4].

The origin, function and fate of reserve cells however is still subject of discussion. In an earlier study on adult uterine cervical epithelium we concluded that the reserve cell population has a stem cell function, based on the presence of p63 and CK 17 in these cells [5]. This stem cell population plays a pivotal role in the process of carcinogenesis of. Since it is suggested to be the target cell for Human Papilloma Virus (HPV) infection [5]. Since cervical carcinoma can be of squamous or of glandular type, the target cell for HPV must be able to differentiate into both directions. In a recent study on human fetal uteri [1] we were able to show that the marker profile of the reserve cells during embryogenesis shows two subpopulations, i.e. a distal CK 17 positive reserve cell population that gives rise to both squamous epithelium and columnar epithelium, and a proximal CK 17 negative subpopulation that gives rise to columnar cells. In the underlying study we tried to identify subpopulations of reserve cells in the adult uterine cervix.

Studies by Carmichael and Jeaffreson [6] and Weikel et al [7] describe the presence of reserve cells in all investigated uterine cervices. The reserve cells were usually most abundant in the upper half of the cervix. This in contrast to Burghardt's [8] observations who described a decreasing number of reserve cells with increasing distance from the squamocolumnar junction.

In this study we focus on the distribution pattern and marker profile of reserve cells along the entire length of the adult endocervical canal. Using a combination of cytokeratin antibodies specific for simple and complex epithelia and the stem cell markers p63, bcl-2 and CK 17. [5;9-11], we aimed at identifying different reserve cell subpopulations in the adult cervix to shed more light on the stem cell function of reserve cells.

Materials and Methods

Tissue specimens and processing

Ten uterine cervices were selected from patients undergoing a hysterectomy for benign indications, such as menstrual cycle disorders or uterine prolapse. Five specimens were from premenopausal women and five from postmenopausal women. Informed consent was obtained and the medical ethical committee of the Deventer Hospital gave its approval for this study.

After measuring dimensions and external inspection, a central sagital section was taken through the entire specimen. This allowed inspection of the endocervical canal, endometrial cavity and the myometrium. More parallel sagital sections were then taken, and in cases with no macroscopic abnormalities a second transverse section was taken approximately 3 mm lateral to the first central section. This section included the ectocervix, endocervix, lower and upper uterine cavity, up to the fundus uteri. This section was formalin fixed and paraffin embedded along with any other sections taken from points of interest. The sections were routinely processed and hematoxylin-eosin (H&E) staining was used for selection of continous epithelium from the ectocervix up to the fundus uteri and to inspect for epithelial abnormalities.

Immunostaining procedures and antibodies.

From the selected tissue blocks 4 μm thick paraffin sections were cut. The first and the last sections were stained for H&E to check that epithelium was present along the entire length of the uterine cavity. If this was the case the unstained slides were subjected to immunohistochemical staining procedures. All slides were immunostained using an automated Ventana Benchmark™ , using the Ventana iView™ DAB detection kit. Four of the cases were also examined after applying different retrieval procedures as described before [1].

Primary antibodies to the following antigens were applied:

p63 (4A4+Y4A3, Neomarkers, Fremont, CA, USA): present in basal/stem cells in epithelial compartments which can differentiate along a squamous pathway (12).

bcl-2 (124, Neomarkers, Fremont, CA, USA) specifically present in cells protected from apoptotic cell death, and usually found in basal/stem cell compartments (10).

cytokeratin 5 (XM-26, Menarini, Athens, Hellas) found particularly in basal cell compartments of squamous and complex epithelia (11).

cytokeratin 7 (OV-TL 12/30, Biogenex, San Ramon, CA, USA) present in glandular cells of amongst others the urogenital tract, while mature squamous epithelia, generally, do not contain cytokeratin 7 (11).

cytokeratin 8 (CAM 5.2, Becton and Dickinson, San Jose, CA, USA) present in glandular cells of most epithelia (13).

cytokeratin 17 (E3, Dakopatts, Glostrup, Denmark) present in basal/stem cells in complex epithelia and occasionally squamous epithelia (11).

Slide evaluation and scoring

The H&E stained slides were used to inspect various morphological features of the epithelial lining of the cervical canal. In each case we evaluated the epithelial lining of the ectocervical squamous epithelium up to the endometrial cavity. In each section the immunostaining was scored in the reserve cells, the endocervical columnar cells and the tubal epithelium for intensity and frequency. Furthermore, we examined the various antibodies for an immunostaining gradient along the entire length of the endocervical canal. The staining results were indepently evaluated by 3 of the authors (J.M, F.S and JW.A). In cases of discrepancy the slides were re-evaluated and consensus was reached in all cases.

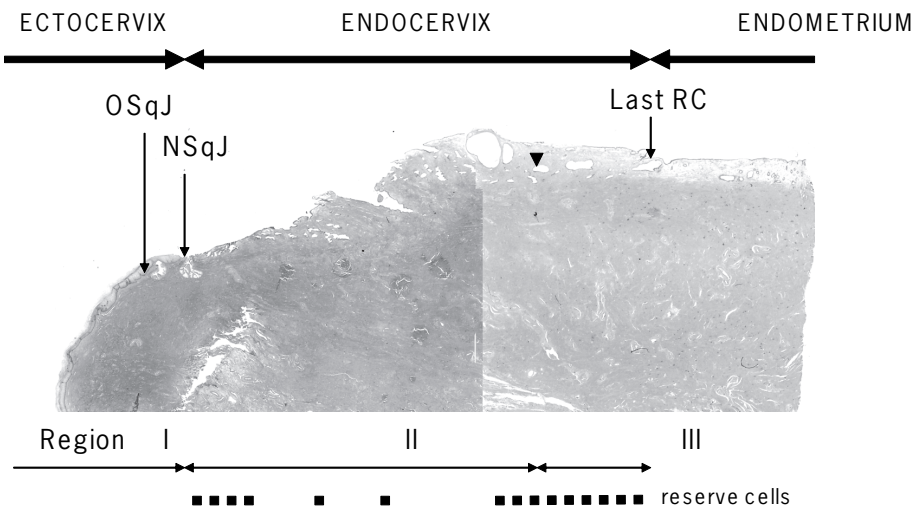
Results

Histological features of the uterine cervical epithelium.

On basis of the histological features encountered in the tissue sections the endocervical canal was subdivided into three regions, each characterized as follows (figure 1):

Region I starts from the ectocervical epithelium and abruptly ends at the functional squamo-columnar junction. From distal to proximal this section is subsequently lined by ectocervical squamous epithelium, mature squamous epithelium, followed by immature squamous epithelium, reserve cell hyperplasia, ending at the new or functional squamo-columnar junction.

Figure 1



Overview of regions I-III of the epithelium of the uterine cervix with the distribution pattern of the reserve cells indicated. Also the original and new squamocolumnar junction (OsqJ and NSqJ), as well as the last reserve cell (rc) and first tubal invagination (arrowhead) are indicated.

Region II starts from the point where the endocervical type of columnar epithelium lines the cervical canal. The lining consist mostly of a single layer of mucin producing columnar cells with basally located nuclei, and sporadic ciliated cells. This epithelium contains foci of subcolumnar reserve cells. The endocervical invaginations in this region are also lined by this endocervical columnar epithelium with sporadic reserve cells.

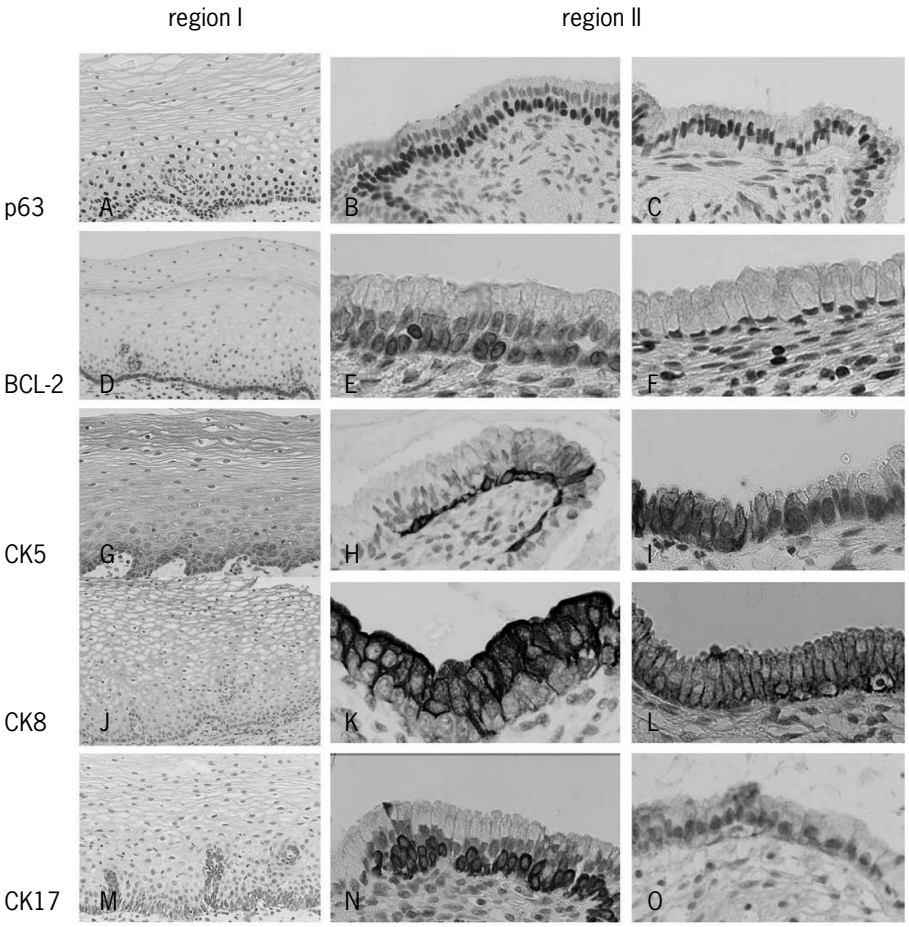
Region III comprises the area where the surface epithelium is still of the endocervical type, but invaginations become lined by tubal epithelium (see arrowhead in figure 1). Eventually all invaginations and the surface are covered by tubal epithelium. Tubal epithelium is defined by the existence of three cell types i.e. ciliated cells, secretory (non-ciliated) cells and thin intercalacted cells [14]. We define the end of the endocervix as the point where the surface epithelium is entirely of the tubal type and no more reserve cells are observed. This epithelium continues up into the endometrial cavity with transitions into the endometroid epithelium.

Distribution pattern of reserve cells.

Reserve cells were present in all ten cervical tissue samples, located beneath the columnar cells lining the endocervical surface, as well as in the invaginations.

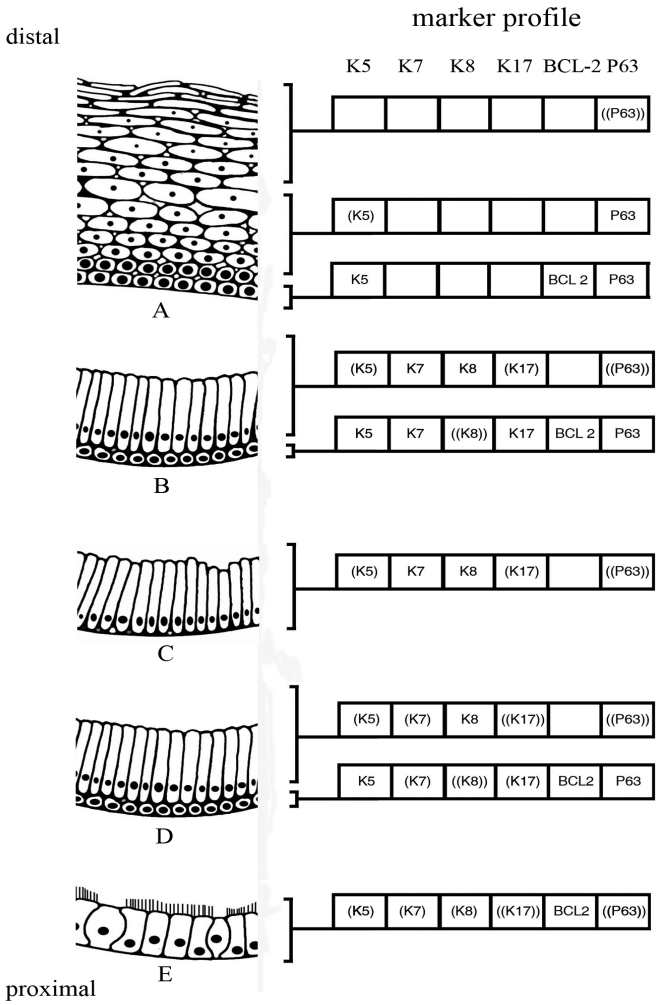
In general a discontinous distribution pattern of subcolumnar reserve cells was observed in regions I-III (see figure 1 for schematic representation). As expected we found reserve cell hyperplasia in region I in areas of squamous metaplasia. Larger streches of reserve cells were usually situated at the proximal part of region II. Strinkingly this was followed by a part of the endocervical columnar epithelium almost no reserve cells. Than, higher in the cervical canal in region III, large stretches of reserve cells were detected. The last reserve cell was always found before the beginning of the endometrial cavity. We never identified reserve cells in the endometrial epithelium.

Figure 2 (figure in color on page 144)



Immunostaining patterns for p63 (A-C), Bcl-2 (D-F), CK5 (G-I), CK8 (J-L) and CK 17 (M-O) of the ectocervical squamous epithelium (A,D,G,J,M), distally located columnar epithelium with underlying reserve cells (B,E,H,K,N) and more proximally located glandular epithelium without underlying reserve cells (C,F,I,L,O).

Figure 3



Schematic overview of the immunomarker profile for CK5, CK7,CK8,CK17, bcl-2 and p63 in the different epithelial cell types from the distal to the proximal regions of the endocervical canal. Annotation in brackets indicates moderate expression and in double brackets indicates weak expression.

A: region I, ectocervical epithelium
B: region II, columnar epithelium with subcolumnar reserve cells
C: region II, columnar epithlium without subcolumnar reserve cells
D: region III, columnar epithelium with subcolumnar reserve cells
E: region III, tubal type epithelium lining the invaginations

Marker study on reserve cells and related epithelia.

Region I

In region I the expression pattern of several layers of the ectocervical epithelium for the different cytokeratins, p63 and bcl-2 was consistent with earlier studies [5;10;15] (figure 2 A,D,G,J,M and figure 3A). The reserve cells in the transitional zone were all positive for p63, bcl2, CK 5, CK 7 and CK 17. Only half of cases showed a 10% staining voor CK 8.

Region II

The subcolumnar reserve cells in this area were all strongly positive for p63, CK 5 and CK 7 (figure 2B, H and figure 3B). Bcl-2 was expressed in most reserve cells (figure 2E and figure 3B). The reserve cells showed weak staining, in less than 5% of the cells, for CK 8 (figure 2K). CK 17 was detected in approximately 80% of the reserve cells (figure 2N).

Endocervical columnar cells were all negative for bcl-2 but showed strong expression for CKs 7 and 8 (figure 2E, F, K, L and fgure 3B and C). Columnar cells in all cases showed some expression for CK 5 (fgure 2H, I), from sporadic to moderate staining intensity. Staining for CK 17 was found in 6 cases within endocervical columnar cells, sporadic staining in 2 cases and moderate staining in 4 cases (figure 2N, O and figure 3B, C). CK 17 positivity was particularly evident in endocervical columnar cells when subcolumnar reserve cells were observed in the same region (figure 2N and figure 3B). Four cases showed no expression for CK 17 in the columnar cells. Furthermore endocervical columnar cells showed sporadic immunoreactivity for p63 (figure 2B, C and figure 3B, C), although the frequency of cells staining was less than 1% and the intensity was variable.

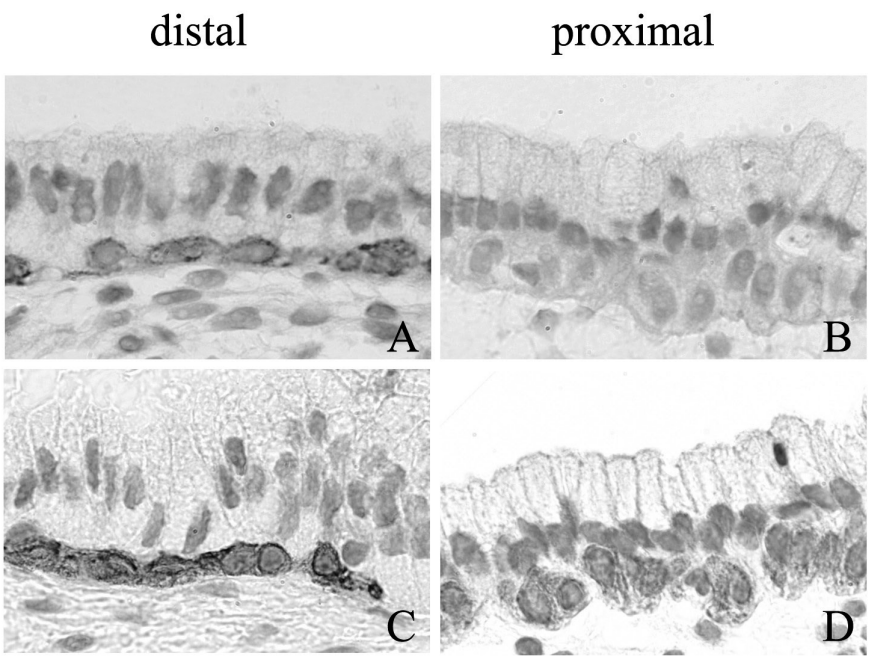
Region III

All reserve cells were strongly positive for p63 and CK 5 and positive for CK 7. CK 8 expression was seen sporadically in only 2 cases (figure 3D). Bcl-2 and CK 17 were present in 80-100% of these reserve cells, although less intense than in region I and II, showing a decreasing gradient of expression from distal to proximal (compare figure 4A and 4B). The bcl-2 gradient was observed using both immunohistochemical detection procedure (see Material and Methods), while the CK 17 gradient became particularly obvious using the automated method (figure 4A and 4B compared with figure 4C and 4D).

The endocervical columnar cells showed a pattern that was slightly different from that in region II. The basal type CK 5 was found in 50% of endocervical cells which overlay reserve cells (figure 3D). The staining patterns for CKs 7,8, 17 and p63 and bcl- 2 were similar to those in region II (figure 3D).

Tubal epithelium (figure 3E) in the invaginations was almost completely negative for p63. Strong bcl-2 staining activity was detected in 60-90% of these tubal cells. CK 7 and CK 8 positive tubal epithelial cells were found with a discontinous patchy distribution pattern. CK 5 was found in 50% of the tubal epithelial cells in 6 cases. The other cases showed a discontinous pattern similar to observations for CK 7 and CK 8. CK 17 showed almost no staining activity in five cases and very weak staining activity in the other five cases.

Figure 4 (figure in color on page 145)



CK 17 expression patterns of distally (A,C) located subcolumnar reserve cells, close to the squamocolumnar junction, and (B,D) proximally located subcolumnar reserve cells in the upper third of the endocervix. Two different immunostaining protocols were applied, i.e. the automated staining procedure (A,B) and a staining protocol with optimized antigen retrieval (C,D).

Discussion

This study focusses on the identification of subpopulations of reserve cells along the uterine cervical canal up to the uterine cavity, based on their distribution pattern and marker profile. We describe the epithelial lining along the entire length of the cervical canal and define the end of the endocervix. We used a well defined set of immunohistochemical markers that enabled us to support our hypothesis of subpopulations of cervical stem cells.

The distribution pattern of the reserve cells we describe confirms previous histochemical studies by Carmichael and Jeaffreson[6] and also Weikel [7]. Like these authors we observed reserve cells in all cervical tissue samples indepent of patient age. We can also confirm their findings on the higher concentration of reserve cells in the upper part of the cervical canal. They also describe a discontinuity in reserve cell distribution and reserve cell patches at a certain distance from the squamocolumnar junction. These findings are in contradiction to the distribution pattern of reserve cells described by Burghardt [16] and Richart [17] who suggest a diminishing frequency of reserve cells as the distance from the squamo-columnar junction increases.

We hypothesize that the concentration of reserve cells in the more distal part of the cervical canal (close to the squamocolumnar junction) attributes to the formation of squamous metaplasia. Reserve cells are used in this metaplastic process, via the pathway of reserve cell hyperplasia which may explain the lower frequency of these cells in this region as compared to the upper part of the endocervix. This theory has also been proposed by Vooijs et al [4;18], who stated that: " the predominant site of pure reserve cell hyperplasia is in the proximal part (region close to the squamocolumnar junction) of the endocervical canal". The higher concentration of reserve cells proximal (in the upper part) in the endocervical canal suggests an exclusive progenitor role for the endocervical columnar cells. From distal to proximal at a certain point the surface is lined by tubal epithelium and almost no reserve cells are found; we define this point as the end of the endocervix. After this point we observed in only 3 cases some reserve cells.

Contrary to the report by O'Connell, we did not find any reserve cells or p63 expression in the lower endometrial region [8]. O'Connell also reports that in the fetus reserve cells are found in the endometrial part of the uterine cavity. It has to be kept in mind however that in the fetus it is not possible to accurately assess the parts of the uterine cavity that will develop either into endometrium or into endocervix [1].

The marker profiles of reserve cells in the vicinity of the squamocolumnar junction demonstrate strong expression for the stem cell markers p63, bcl-2 and squamous cell markers CK 5 and CK 17 as well as the luminal cell marker CK 7. Expression of both the luminal and basal markers could be considered proof for our hypothesis that reserve cells are capable of dual differentiation. On the one hand they are capable of differentiating into a squamous type epithelium, during which process they will loose the expression of CK 7, on the other they have the capacity to differentiate into columnar cells and than loose expression of CK 5 and CK 17. The lower levels of CK 17 expression in the more proximal region of the endocervix indicates that this subpopulation of reserve cells are predestined to replenish the columnar cell compartment and have lost their ability to undergo squamous differentiation.

The expression pattern of the columnar cells provides more proof for this hypothesis. We observed CK 5 expression and CK 17 expression, albeit less frequently, in endocervical columnar cells lying above reserve cells or adjacent to them. We interpret this remarkable expression of CK 5 and CK 17 in these endocervical cells as proof that they have developed from the underlying reserve cells.

In conclusion we found a discontinuous distribution pattern of reserve cells in all the uterine cervix with concentrations close to the squamocolumnar junction and in the upper third of the endocervical canal. In the middle part of the cervices only sporadic reserve cells were observed. Based on their marker profile we define two subpopulations of reserve cells in the adult human uterine cervical epithelium; i.e. a CK 17 positive subpopulation in the lower part of the cervical canal having a stem cell function for the squamous and columnar epithelium, and a subpopulation of CK 17 negative reserve cells having only a stem cell function for only columnar cells.

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5

Glutathione S-Transferase π is expressed in (pre) neoplastic lesions of the human uterine cervix irrespective of their degree of severity

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Frans C.S. Ramaekers.

Based on:
Anticancer Research 1997; (17) 4305-4310

Abstract

Introduction

Glutathione S-transferase π (GST π) is involved in a variety of cell detoxification processes. In the uterine cervix its presence has been associated with high grade cervical intraepithelial neoplasia (CIN), but the reports are conflicting. For this reason we immunohistochemically investigated glutathione S-transferase π expression in a well documented sequence, leading to cervical cancer.

Materials and methods

The series of tissue samples that were examined comprised normal, metaplastic, dysplastic (CIN I, II and III) and malignant cervix. GST π expression was examined in 15 cases of uterine cervix lined with normal epithelia, in 11 cases of CIN I, 9 cases of CIN II, 10 cases of CIN III, 6-cases of squamous cell cervical carcinomas and 5 cases of adenocarcinoma of the cervix.

Results

Both nuclear and cytoplasmic staining reactions were noted. In normal ectocervical epithelia a moderately strong nuclear and cytoplasmic staining reaction was noted, while in immature squamous metaplasia staining was more intense. Only 50% of the endocervical cells were immunostained while almost 100% of the reserve cells stained moderately positive, mainly restricted to the cytoplasm. Irrespective of severity, CIN lesions showed a moderate staining intensity in both cytoplasm and nuclei. Cervical carcinoma, irrespective of their type, showed significantly less staining activity.

Conclusion

GST π occurs in normal cervical epithelium and in all stages of premalignant cervix, suggesting an important role in the detoxification process in all these stages. All reserve

cells stained positive for GST π suggesting that these cells need special protection against influences from outside. The ubiquitous presence of GST π indicates, in contrast to the earlier reports, that the enzyme does not play a crucial role in the initiation of the carcinogenic cascade. However, the absence of this detoxificating enzyme in the nucleus of the majority of cervical carcinomas may indicate that xenobiotic compounds are not catabolized and may therefore exert their mutagenic activity, resulting in tumor progression.

Introduction

In the uterine cervix well defined premalignant conditions may develop into cervical carcinoma [1]. Most cervical intraepithelial lesions (CIN) will, however, not progress to cervical carcinoma [2]. It is generally thought that low grade lesions, i.e. CIN I and CIN II, have a low progressive potential and if left untreated probably less than 10% of the cases would progress. In high grade lesions (CIN III) up to 50% are thought to progress to cervical carcinoma when not treated [1]. It is generally believed that non-progressive CIN lesions may remain stable for years or even regress and eventually disappear.

On the basis of morphologic criteria it is relatively easy to distinguish the different grades of CIN. It is, however, not possible to predict whether an individual CIN lesion is progressive, stable or regressive in nature [2].

Application of molecular techniques in this field has provided new insight into the cellular processes that are involved in determining the malignant nature of cervical tissue. In the tumorigenesis of cervical carcinoma a number of toxic substances has been implicated, and for this reason a number of studies have examined the expression of glutathione S-transferase π (GST π) in CIN [3-7]. GTS π is a member of a multigene enzyme family which is found mainly in the cytoplasm, and has been shown to have a detoxificating capacity for electrophilic compounds, including carcinogens [8;9]. The first study by Shiratori et al [3] reported increased immunohistochemical staining for GST π with increasing grade of CIN. This was in accord with the results of Zhang and co workers [10] who demonstrated a close correlation between the intensity of immunohistochemical staining with GST π and increasing grade of preneoplasia in oral squamous epithelia. These results were not confirmed in later studies on the cervix [5-8]. Because results from studies investigating GST π expression in cervical epithelia of various degrees of (pre)neoplasia are conflicting, we investigated the presence of GST π in a well defined set of normal, metaplastic, dysplastic and malignant cervical lesions, in an effort to improve our understanding of cervical carcinogenesis.

The role of the reserve cell in carcinogenesis is very interesting. We presume the reserve cell to be the target cell for HPV infection [11]. From the point of carcinogenic theories involving the stem cell of the epithelium we hypothesized a subpopulation of reserve cells being the progenitor cell of the cervical epithelium [11;12]. This was the reason to focus on the expression of GST π in reserve cells.

Materials and Methods

Tissue specimens

Formalin fixed and paraffin embedded uterine cervix specimens were used in this study. As controls, excision samples were taken from hysterectomy specimens removed, for benign conditions in premenopausal women (n=15). Histologic diagnosis was performed on H&E stained slides. In the 15 normal specimens ectocervical epithelium was diagnosed in 9 cases, endocervical columnar epithelium in 14 cases, reserve cells in 7 cases, mature squamous metaplasia in 8 cases, immature squamous metaplasia in 4 cases. Diathermy loop excision specimens were taken from women with cytologically verified dysplasia. The samples showed CIN I in 9 cases, CIN II in 11 cases and CIN III in 10 cases. In these slides normal epithelia were also detected, with ectocervical epithelium found in 6 sample, endocervical columnar epithelium in 22 samples, reserve cells in 9 samples, mature squamous metaplasia in 16 samples and immature squamous metaplasia in 3 samples. The tissue samples representing cervical carcinoma were taken from hysterectomy specimens and included 7 cases of non-keratinizing squamous cell carcinoma and 6 cases of adenocarcinoma.

Immunostaining protocol

Two micron thick sections were cut from the representative paraffin blocks, mounted on glutaraldehyde activated slides and dried overnight at 56°C. After deparaffinazation and blocking of endogenous peroxidase activity with 0.3% H_2O_2 the slides were incubated with normal goat serum to block non-specific binding sites. This was followed by a 1 hour incubation with the polyclonal GST π antibody (BioGenex, San Ramon, California, USA) at room temperature. After three subsequent washing steps with phosphate buffered saline (PBS) at pH 7.4, antibody binding was detected with the SuperSensitive Biotin-StreptAvidin system (BioGenex) and detected with diaminobenzidine (DAB) as chromogenic substrate. The sections were briefly counterstained with haematoxylin and mounted with Coverfilm. In negative controls the primary antibody was omitted. Malignant and benign breast tissues were used as positive controls for cytoplasmic as well as nuclear reaction of GST π.

Evaluation of staining reactions

The staining results were evaluated by two of the authors (JM and FS). In cases of discrepancy the slides were reviewed together and consensus was reached in all cases. Immunostaining intensity was scored as negative, weak, moderate or strong. The number of positively staining cells was semiquantitively evaluated, with four groups being distinguished, i.e. cases with 1 to 25%, 25-50%, 50-75% and 75-100% of the cells positive.

Cytoplasmic and nuclear staining reactions were separately scored, while also the localisation of the staining reaction within the epithelial layers were scored, Therefore CIN lesions were subdivided into three compartments, i.e. basal, intermediate and superficial, each consisting approximately one-third of the epithelial thickness. Also staining of non-epithelial tissues was evaluated.

Results

The immunohistochemical staining results for GST π are schematically presented in Figure 1A and 1B and illustrated Figure 2. For the sake of clarity we only describe the most salient features.

Normal cervical epithelia

Cytoplasmic staining of moderate intensity was observed in all cases of ectocervical non-keratinizing epithelium, through the full epithelial thickness (figure 1A). Nuclear staining was located in the parabasal and intermediate layers (figure 1B and 2a). The cytoplasm of endocervical columnar cells stained moderately strongly in 50% of cases while in almost all cases a moderate nuclear staining reaction was observed (figure 2b). In all reserve cells the cytoplasm stained moderately positive, with only 40% of the nuclei showing a moderate immunostaining reaction (figure 2b). Full thickness cytoplasmic staining was observed in mature squamous metaplasia with the most intense staining reaction in the intermediate cell layer (figure 1A). Nuclear staining was only observed in 50% of cases in the parabasal and intermediate layers and almost no nuclear staining reactivity was seen in the basal and superficial cell compartments (figure 1B). Moderate to intense full thickness staining was observed in the cytoplasm of cells in immature squamous metaplasia while an intense nuclear staining reaction was observed in 50% of the cases (figure 2c).

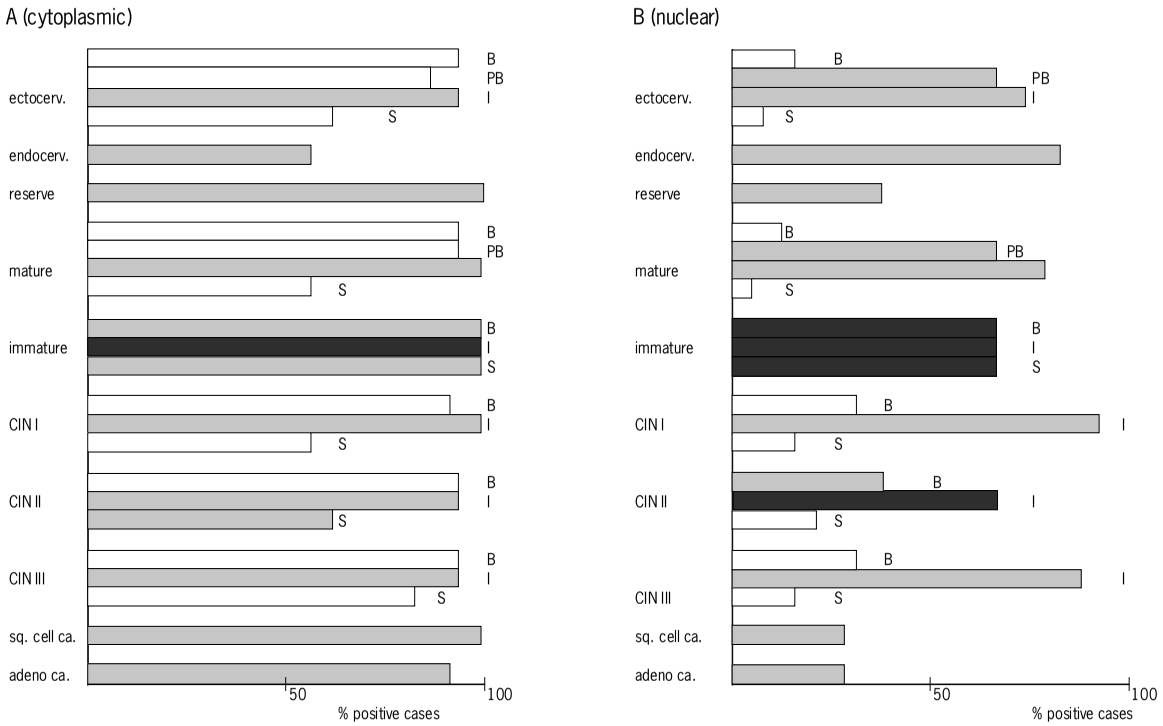
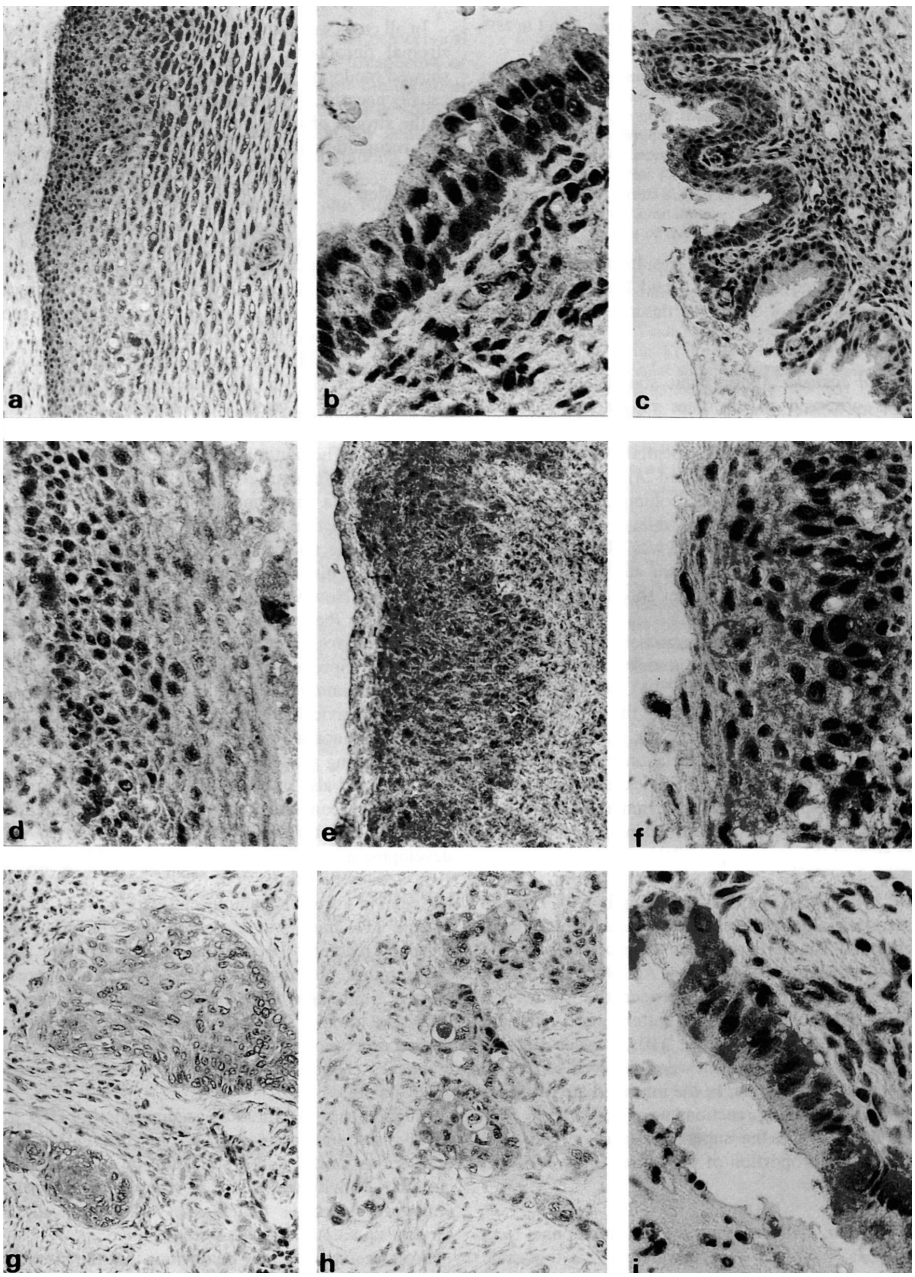


Figure 1

Schematic representation of GST π immunostaining reactions in epithelial tissues of the uterine cervix comprising ectocervical epithelium (ectocerv.), endocervical epithelium (endocerv.), reserve cells (reserve), mature squamous epithelium (mature), immature squamous metaplastic epithelium (immature), CIN I, CIN II, CIN III, squamous cell carcinoma (sq.cell ca.) and adenocarcinoma (adeno ca.). The bars represent the percentages of cases with immunoreactivity in cells with the GST π antibody. White bars indicate a weak staining reaction, gray bars indicate a moderate intensity and the black bars indicate strong reactivity. (A) cytoplasmic staining (B) nuclear staining Abbrevations: B basal cell compartment; PB parabasal cell compartment; I intermediate cell compartment; S superficial cell compartment.

Figure 2 (figure in color on page 146)



GST π immunostaining reactivity in ectocervical epithelium (a), endocervical epithelium and reserve cells (b), immature squamous metaplasia (c), CIN I (d), CIN II (e), CIN III (f), squamous cell carcinoma (g), adenocarcinoma (h) and tubal metaplasia (i).

Preneoplastic conditions

Cytoplasmic staining in CIN I, II and III was moderate in almost all cells in the basal and intermediate epithelial cell compartments and in most cells in the superficial cell compartment (figure 1A). Nuclear staining reactions showed slight differences between the various cell compartments, although the reaction pattern was almost the same in all grades of CIN (figure 1B). Nuclear staining reactions in the basal compartment were observed in 30% of cases irrespective of CIN grade. In the intermediate cell layer moderate to strong staining reactions were observed in 50% to 100% of nuclei while the superficial cells only showed reactivity in a small proportion of the nuclei (figure 2d, 2e, 2f).

Cervical carcinoma

All squamous cell carcinomas and adenocarcinomas showed a moderate cytoplasmic immunoreactivity. Nuclear staining of moderate intensity was observed in approximately 30% of the cases (figure 2g, 2h). In all cases weak immunoreactivity was focally found in the stromal fibroblasts. Smooth muscle cells in blood vessels showed moderate immunostaining and endothelial cells were usually negative. A striking cytoplasmic staining was observed in the stroma of tubal metaplasia (figure 2i), which allowed easy recognition of this epithelial tissue type.

Discussion

In the present study the role of the placental form of Glutathione S-transferase π (GST π) in the initiation and progression of human cervical neoplasia was investigated, using well defined tissue samples representing the subsequent steps in the process of cervix carcinogenesis. Special attention was given to the expression of GST π in the reserve cell, probably the stem cell of the cervical epithelium.

Previously, Shiratori et al [3] demonstrated increased expression of GST π, both nuclear and cytoplasmic, in all grades of CIN as well as in invasive cervical lesions, as compared to the absence of expression in normal ectocervical epithelium. These authors therefore suggested that GST π could be a useful marker for (pre)neoplasia. Also Randall et al [7], found a more intense nuclear staining activity in a greater proportion of the cells of all stages of CIN, and staining of the nuclei in the upper epithelial layers of these lesions was noted, as compared to normal ectocervical epithelium. However, these authors [7] stressed the presence of GST π in the cytoplasm of normal cells and in cells of all stages of cervical tumorigenesis.

In a comprehensive study by de Camargo et al [6] cytoplasmic immunoreactivity was also noted in the majority of normal epithelia as well as in dysplasia, CIN and cervical carcinoma. An increase in nuclear staining intensity with increasing grade of CIN, like Shiratori et al [3] demonstrated, could also not be confirmed by de Camargo et al [6]. The authors concluded that GST π might be related to an increased cell turnover, but is not related to neoplastic development.

In a study by Carder et al [5] the same pattern of staining was seen in normal squamous epithelium as in CIN I. In CIN II and III, there was increased staining for GST π, with nuclear reactivity being particularly more prominent. Furthermore, all squamous cell carcinomas were strongly positive, although there was some heterogeneity within individual tumors. The authors [5] showed, however, that this altered expression of GST π is not specific for dysplasia, since identical changes were seen in non-dysplastic viral condylomata.

Maguire et al [4] found an accumulation of GST π in most CIN lesions and carcinomas, but no differentiation between the various grades and types could be made. Furthermore, immunoreactivity was noted in reserve cell hyperplasia and immature squamous metaplasia, while practically all ectocervical squamous epithelia were negative.

From the above, it may be obvious that the literature is full of discrepancies concerning the expression of GST π in the tumorigenic process. We could show that the cytoplasmic activity of GST π is seen in all layers of normal ectocervical epithelium and also in all cell layers of CIN irrespective of grade. The staining intensity varied slightly for the different cell layers. Most striking was the strong cytoplasmic staining intensity in immature squamous metaplasia. In general a lower percentage of cases showed nuclear staining activity than cytoplasmic staining activity. The nuclear staining activity was concentrated in the parabasal and intermediate cell layers in all tissue types. Nuclear staining was again most intense in immature squamous metaplasia. For squamous cell carcinoma and adenocarcinoma of the cervix we found nuclear immunoreactivity in a significantly lower percentage of the cases as compared to the normal epithelia and preneoplastic lesions. These observations are in striking contrast to those of Shiratori et al [3], and in line with the studies of Randall et al [7] who showed variable, and in general less nuclear GST π immunostaining in cervical carcinoma as compared to CIN.

In a recent study by Moskaluk et al [13], a drastic down-regulation of GST π are involved in the initiation of cervical carcinogenesis.

In conclusion we would like to state that it is unlikely that changes in the level of GST π are involved in the initiation of cervical carcinogenesis. However, the absence of this detoxificating enzyme in the nucleus of the majority of cervical carcinomas may indicate that xenobiotic compounds are not catabolized and may therefore exert their mutagenic activity, resulting in tumor progression. The strong, cytoplasmatic, expression of GST π in reserve cells supports the hypothesis that subpopulations of reserve cells can be the progenitor cell of the cervical epithelium.

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6

Can Cytokeratin 8 and 17 immunohistochemistry be of diagnostic value in cervical cytology? A feasibility study.

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Cancer (Cancer Cytopathology) 1999;(87) 87-92

Abstract

Introduction

Based on results from evaluation of tissue sections from premalignant lesions of the uterine cervix, the authors examined the hypothesis that immunostaining of Papanicolaou-stained cytologic smears with monoclonal antibodies to cytokeratins 8 and 17 allows detection of cervical intraepithelial neoplasia (CIN) with progressive potential. They also investigated whether detection of these two keratin subtypes could be of help in the analysis of normal and/or poor quality cytology smears.

Methods

Sixty-one Papanicolaou-stained smears, representing 25 normal smears, 8 CIN I, 7 CIN II, 18 CIN III, and 3 cervical carcinomas, were stained with CAM 5.2 and E3, which are capable of detecting cytokeratin 8 and 17, respectively. The percentages of immunoreactive normal, metaplastic, dysplastic, and malignant epithelial cells were determined.

Results

In normal cervical smears, cytokeratin 8 was detected in endocervical columnar cells and sporadically in immature squamous metaplastic cells. Cytokeratin 17 was identified in reserve cells and frequently in immature squamous metaplastic cells. In CIN, the number of cases in which cytokeratin 8 was present increased with the severity of the lesion. Cytokeratin 17 was found in the majority of CIN lesions, irrespective of grade. Intensity of immunostaining and number of cells stained per lesion varied and were also not related to the severity of CIN.

Conclusion

The use of the cytokeratin 8 antibody in normal cervical smears enabled the detection of endocervical cells in cases where they were thought to be absent, particularly in cases with severe inflammation. Staining with cytokeratin 17 enabled the identification of reserve cells or immature metaplastic cells, which were often misinterpreted as parabasal cells. The application of antibodies to these subtypes of keratins in cervical cytology can to a certain extent help in the identification of CIN and may in future be tested in automated screening.

Introduction

Keratin phenotyping of normal, premalignant, and malignant epithelium of the uterine cervix has revealed interesting changes in the expression patterns of cytokeratins 8 and 17 on progression of cervical intraepithelial neoplasia (CIN) [1-8]. In tissue studies, cytokeratin 8 was found to occur in normal endocervical columnar cells but not in ectocervical squamous epithelium [3-5;7-9]. It was found to display a maturation-related expression pattern in immature squamous metaplastic epithelium, being absent in more mature or fully matured squamous metaplastic epithelium. In formalin fixed, paraffin embedded tissues, the number of lesions expressing cytokeratin 8 was less than observed in fresh frozen tissue specimens [1;7]. Based on observations in fresh frozen tissue, cytokeratin 8 was detected in a minority of CIN I and CIN II lesions and in approximately 80% of CIN III lesions. This observation prompted the hypothesis that the persistent presence of cytokeratin 8 in a CIN lesion indicated that this lesion was progressive in nature [2;4;5]. This hypothesis was supported by the observation that cytokeratin 8 was invariably present in cervical carcinomas [7;10]. Cytokeratin 17 was found in endocervical reserve cells and in immature squamous metaplastic epithelium, but it was absent in endocervical columnar cells, ectocervical squamous epithelium, and mature squamous metaplastic epithelium. It is noteworthy that with increasing severity of CIN, cytokeratin 17 was more frequently found and the intensity of immunostaining was also increased. In cervical carcinoma, cytokeratin 17 was always detected [7]. These observations prompted us to propose the theory that the combined presence of cytokeratin 8 and 17 in CIN was reflection of its progressive potential. Based on these findings, we investigated whether immunostaining of Papanicolaou stained cytology smears with monoclonal antibodies to cytokeratin 8 and 17 could be used to detect CIN in these specimens. Furthermore, we examined whether or not cytokeratin phenotyping of cervical smears could contribute to the accuracy of cytodiagnosis and to our understanding of the pathogenesis of CIN and cervical carcinoma.

Material and Methods

Cytologic Specimens

The cytologic material used in this study had been stored for 11-13 years and was retrieved from the archives of the Department of Pathology at the SSDZ/ Reinier De Graaf Group, Delft, The Netherlands. It comprised 25 Papanicolaou-stained cervical smears with no abnormalities, 8 smears with cytologic findings consistent with CIN I, 7 smears with CIN II, 18 with CIN III, and 3 with cervical squamous cell carcinoma. Cases with reasonable numbers of diagnostic cells were selected.

Antibodies

CAM 5.2 (IgG2a) was supplied by Becton Dickinson (San Jose, CA, USA). In immunoblotting studies, CAM 5.2 was shown to be reactive with cytokeratin 8 and to a minor degree with cytokeratin 7 [5], but not with cytokeratins 18 and 19 [2], as suggested previously. In tissue studies it reacts with columnar cells of the endocervix. CAM 5.2 does not stain keratinizing or nonkeratinizing squamous metaplastic epithelium. E3 (IgG 2b) was supplied by DAKO A/S (Glostrup, Denmark). The E3 cytokeratin antibody is specific for cytokeratin 17. It reacts with endocervical reserve cells and also with immature squamous metaplastic epithelium.

The primary monoclonal antibodies were tested on separate "normal" cervical smears to determine the optimal antibody dilutions, which were 1:400 for CAM 5.2 and 1:4 for E3.

Staining Procedure

All smears used in this study were Papanicolaou stained (hematoxylin, Orange G, E50, Merck, Germany) for routine cervical screening at least 10 years prior to retrieval. In the current study the smears were all rescreened. Areas of the smear containing reserve cells, squamous metaplastic cells, and columnar cells were marked on the coverslip, as were dysplastic cell groups in smears consistent with CIN. Photocopies were taken of the slide, allowing relocation of the marked groups after immunohistochemical staining procedures had been performed.

The coverslips were removed by immersing the slides in xylol for at least 24 hours. In some cases this process was accelerated by a microwave step for 2 minutes at 700W. Each slide was then divided in half by a paraffin bar across the middle of the slide perpendicular to the long axis. In this way two separate parts of the original smear could be investigated.

Cells were rehydrated in a descending alcohol series (95%, 70% and 50%), after which they were rinsed in distilled water for 10 minutes and phosphate-buffered saline (PBS, pH 7.4) at room temperature. Each slide was then incubated for 10 minutes in 5% bovine serum albumin (Sigma, St. Louise, MO) in PBS. One half of the slide was incubated with CAM 5.2 and the other half with E3.

The immunostaining procedure was as follows: Step1, incubation with the appropriately diluted primary cytokeratin antibody for 1 hour at 37° C; Step 2, rinsing in PBS; Step 3, incubation with biotinylated rabbit antimouse IgG, containing 2 mL Tris HCl buffer, 20 µL streptavidin and biotinylated horseradish peroxidase dilution 1:600 for 30 minutes; Step 4, rinsing in PBS; Step 5, rinsing in tap water; Step 6, incubation with 3-amino-9-ethylcarbozol AEC (0.5 mg/mL Sigma); Step 7, rinsing in tap water for 30 minutes; Step 8, counterstaining with haematoxylin, a repeat rinsing, and coverslip with glycerine/gelatine. Marks were then replaced on the slides with use of the photocopies. The level of immunostaining was evaluated by counting the number of immunostained cells and the total number of cells per epithelial cell type. In this way percentages of metaplastic, dysplastic, and neoplastic cells immunoreactive for the two antibodies were determined.

Results

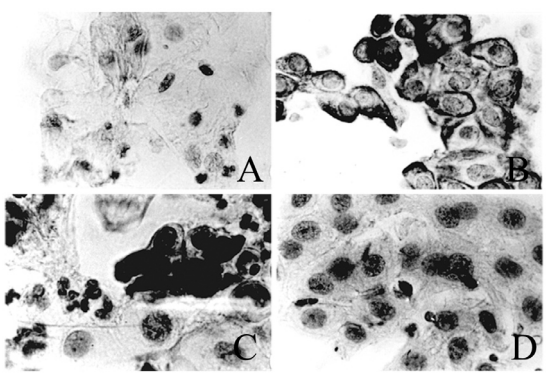
Normal Cervical Epithelium Cells in Cytology Smears

Results for normal cervical epithelial cells are given in Table 1. Cytokeratin 8 was not detected in ectocervical squamous epithelial cells (Fig. 1a) In 2 cases a few superficial ectocervical cells stained weakly with the cytokeratin 17 antibody. Strong immunoreactivity was served with CAM 5.2 in both cell sheets and in dispersed endocervical columnar cells (Fig. 1b). Groups of endocervical columnar cells present in inflammatory infiltrates, which could not be classified previously, showed intense cytokeratin 8 staining that highlighted their external contours and enabled identification (Fig. 1b).

Table 1. Expression of cytokeratins 8 and 17 in different cell types in normal cervical smears.

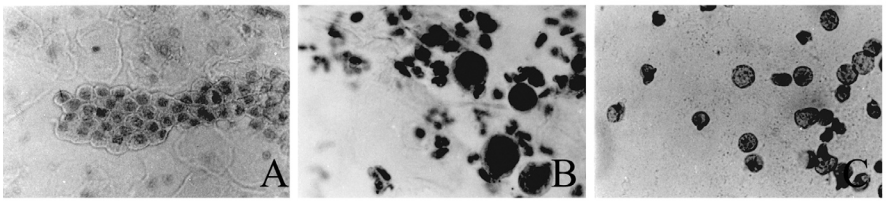
	Keratin 8	Keratin 17	Keratin 8 and 17
Ectocervical squamous epithelial cells	0/25[a]	2/25[a]	0/25[a]
Endorcervical columnar cells	0/25	0/25	0/25
Reserve cells	4/4	4/4	4/4
Squamous metaplastic cells	3/21	10/21	2/21
a. number of positive cases over the total number of cases tested.			

Figure 1



Immunoperoxidase of Papanicolaou-prestained slides with ectocervical squamous cells (A), endocervical columnar cells (B), reserve cells (C), and immature squamous metaplastic cells (D) are shown after staining for keratin 8.

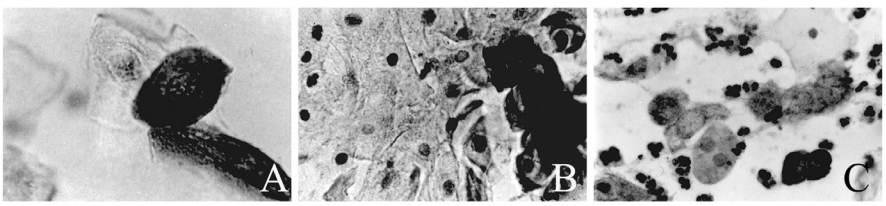
Figure 2



Immunoperoxidase staining patterns of Papanicolaou-prestained slides with ectocervical squamous cells (A), reserve cells (B) and naked nuclei (C) shown after staining for keratin 17.

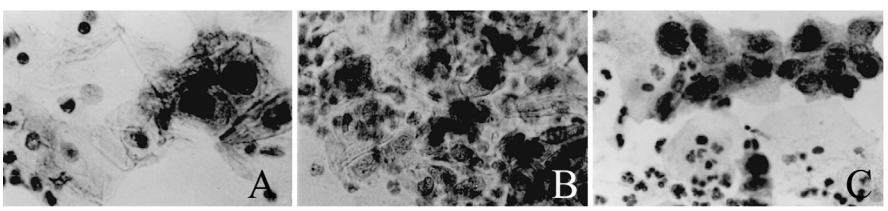
Without exception, these cells were negative for the cytokeratin 17 antibody (Fig. 2a). In four slides reserve cells were found. Both the cytokeratin 8 antibody (Fig. 1c) and the cytokeratin 17 antibody (Fig. 2b) were strongly expressed in these cases. Naked nuclei, classified by Boon et al [11] as reserve cells, demonstrated no immunoreactivity with either antibody (Fig. 2c). Squamous metaplastic cells were found in 21 smears. Cytokeratin 8 was detected in some dispersed cells in 2 cases (Fig. 1d), and cytokeratin 17 antibody stained scattered cells in 10 cases with moderate intensity.

Figure 3



Immunoperoxidase staining patterns of Papanicolaou-prestained slides with cervical intraepithelial neoplasia CIN I (A), CIN II (B), CIN III (C) are shown after staining for keratin 8.

Figure 4



Immunoperoxidase staining patterns of Papanicolaou-prestained slides with cervical intraepithelial neoplasia CIN I (A), CIN II (B), CIN III (C) are shown after staining for keratin 17.

Cervical Intraepithelial Neoplasia

Results for CIN are given in Table 2. Of the 8 cases with CIN I, 4 displayed cytokeratin 8 positivity in approximately 10% of the cells (Fig. 3a) and 5 showed immunoreactivity for cytokeratin 17 (fig. 4a), with approximately 60% of the cells positive. Intensity of staining for both antibodies was mild to moderate.

In CIN II cytokeratin 8 was expressed in 4 of 7 cases (Fig. 3b), in which an average of 15% of cells were positive with moderate intensity. The cytokeratin 17 antibody stained cells in 6 of 7 cases; 40% of cells stained intensely (Fig. 4b). Twelve of the 18 smears with CIN III were immunoreactive for cytokeratin 8 (Fig. 3c); approximately 10% of cells stained with mild-to-moderate intensity. Fourteen of 17 cases were immunoreactive for cytokeratin 17 (fig. 4c), with an average of 25% of cells intensely immunoreactive.

Table 2. Expression of cytokeratins 8 and 17 in different cell types in normal cervical smears.

	Keratin 8		Keratin 17		Keratin 8 and 17
CIN I	4/8 [a]	10% [b+c]	5/8 [b+c]	60% [b+c]	2/8 [a]
CIN II	4/7	15% ++	6/7	40% +++	3/7
CIN III	12/18	10% +	14/17	25% +++	6/12
Cervical squamous cell carcinoma	2/3	25% +++	2/3	25% +++	1/3
a. number of positive cases over the total number of cases tested.					
b. % of positive cells.					
c. Intensity of immunoreaction: + weak, ++ moderate, +++ strong.					

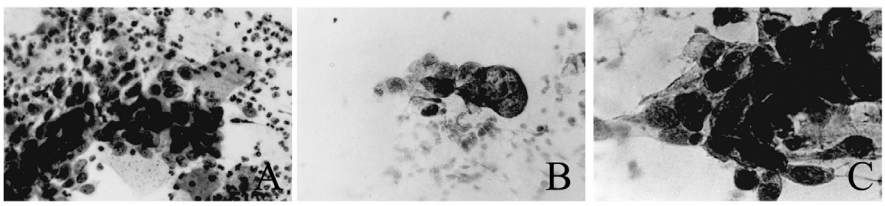
Squamous Cell Carcinoma

These results are shown in Table 2 and Figure 5. Cytokeratin 8 was detected in 2 of the 3 cases of squamous cell carcinoma. Approximately 25% of the cells stained intensely (Fig. 5b). Cytokeratin 17 stained a similar percentage of cells in the same cases (Fig. 3c).

Combined Results of Cytokeratin 8 and 17 Immunostaining

In approximately 25% of the cases of CIN I, CIN II, and CIN III, both antibodies were expressed.

Figure 5



Immunoperoxidase staining patterns of malignant cells from squamous cell carcinoma of the cervix are shown in the original Papanicolaou-stained slide (A) and after staining with keratin 8 (B) and keratin 17 (C).

Discussion

Comparison of Cytokeratin Expression Patterns in Cervical Cytology Smears and in Cervical Tissue Sections

The monoclonal antibodies to cytokeratins 8 and 17 used in this study gave excellent staining results in routinely Papanicolaou-stained cervical smears that had been stored for longer than 10 years. Antigen retrieval procedures were not necessary, and we did not observe any improvement in staining results when we compared the slides that had been subjected to micro irradiation (in order to remove the coverslip) with smears in which this treatment was not necessary. Background staining was completely absent, even in inflammatory exudates.
When we compared the cytokeratin expression patterns in cytologic specimens with those previously observed in tissue slides, a number of differences were observed. Cytokeratin 8 expression in tissue specimens with CIN I was observed as very minor staining in about 10% of cases, whereas cytologic smears harbouring CIN I stained in 50% of cases. In cytologic smears harbouring CIN II, the number of cases that stained for cytokeratin 8 had slightly increased compared with the smears form CIN I lesions; and again, compared with histologic sections, the percentage of cases staining was very much higher. In cytologic smears with CIN III 67% of cases expressed cytokeratin 8, with many cells displaying moderate staining intensity, whereas in histologic sections 80% of cases staining. Again, cytokeratin 17 expression did not significantly increase with the severity of CIN in the smears.

In contrast to previous observations regarding histologic specimens, in this study the number of CIN lesions showing simultaneous expression of cytokeratin 8 and 17 in smears did not show the same dramatic increase with severity of CIN, as approximately 50% of the positive cases were positive for both antibodies irrespective of CIN grade.

On the basis of histologic studies, we and other authors have suggested that expression of cytokeratin 8 alone or in combination with cytokeratin 17 in CIN may signify that a group of lesions could be more aggressive and develop into cervical carcinoma [1;2;5;10]. This was supported by the observation that low grade CIN expressed these cytokeratins in small

numbers of cases, whereas in high grade CIN large numbers of cases expressed these two cytokeratins. Additional proof was based on the fact that cytokeratin expression is generally conserved during neoplastic development. This also seems to be the case for cervical carcinoma, which expresses cytokeratins 8 and 17 irrespective of type and grade.

The fact that the percentage of cytologic specimens combining cytokeratins 8 and 17 is higher than the positive fraction estimated from tissue section studies could indicate a relatively high percentage of progression of low grade CIN lesions, as has been suggested by Whittaker et al [12]. However, it is generally thought that only a low percentage of low grade CIN lesions are progressive in nature [13]. The immunohistochemistry protocol we used may have influenced our results. As we did not use an antigen retrieval technique for the cytologic specimens, one could expect immunoreactivity to be even more intense if heat-induced epitope retrieval was used; however, immunoreactivity in cytology specimens is often excellent without a retrieval step [14].

We observed that cytokeratin immunostaining allowed better insight into the cellular components of a cervical smear. For example, parts of the smear that showed numerous inflammatory cells exhibited groups of cells that could not be categorized in Papanicolaou-stained specimens. These cells showed an intense reactivity with cytokeratin 8 close to their membranes. This made identifying endocervical cells easy. Small cells with relatively large nuclei and a thin cytoplasmic rim that displayed beginning squamoid characteristics could be identified as reserve cells or very immature squamous cells on the basis of their cytokeratin 17 expression. Naked nuclei, identified by Boon as reserve cells [11], displayed no immunoreactivity at all. The cytoplasm of these cells was undoubtedly stripped, and this could of course explain their lack of immunoreactivity. On the other hand, it could also be expected that at least some of these cells had some stainable cytoplasm. We consider these nonimmunoreactive "cells" to be naked nuclei of different cell types from the cervix that may have lost their cytoplasm during smear-taking or processing.

Future Prospects

Cervical cytodiagnostics urgently need specific markers capable of distinguishing between dysplastic and normal epithelial cells, as well as markers capable of distinguishing progressive CIN lesions from those with more indolent behaviour. Studies of other types of (pre)malignancies provide indications that antibodies to the so-called "simple cytokeratins", such as cytokeratin 8, could become such markers. Lane et al [15]. Reported that when cytokeratin 8 positive cells were detected in a smear from the oral cavity, this invariably indicated the presence of a carcinoma of the oral cavity. To study the prognostic value of cytokeratins 8 and 17 in the uterine cervix, cytology expression wil have to be examined in successive cervical smears from women with progressive CIN lesions. Only this approach will relate the expression of keratins 8 and 17 to the prognoses of patients with abnormalities of the cervix.

Cytokeratin 8 is found in a large number of dysplastic lesions, but it is also detected in some reserve cells, some cases of immature squamous metaplastic epithelium, and all endocervical cells. Usually these normal cells are easily identified, thus allowing slides with "no abnormalities" that express cytokeratin 8 to be separated form slides showing CIN. Even then, however, a large number of cases of CIN (approximately 40%) are not immunoreactive, limiting the applicability of this method. Automated screening may benefit from case selection on the basis of the method described above. The cytokeratin 17 antibody recognizes in a high number of cases with CIN lesions, but approximately 20-30% of normal cells will be recognized as false-positive and a number of CIN lesions will not stain. Obviously this percentage is far too high, but when cytokeratin 17 positivity is used as an indicator for manual rescreening of the specimens it may be acceptable, and it would therefore be interesting to investigate the applicability of this method.

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7

General discussion

General discussion

Invasive cervical cancer develops from the different stages of intraepithelial neoplasia (CIN) [1]. The development of cervical neoplasia is the result of proliferation by high risk, oncogenic human papillomavirus (HPV) transformed cells. Untill now it has not been possible to identify and analyze these early infected cells or cell populations [2-4], which must originate from an epithelial progenitor or stem cell like in other types of epithelial cancer [5-7]. This cancer stem cell hypothesis represents a relative new concept in cancer biology, and research on cancer stem cells now runs in parallel with the research on normal embryonic and adult stem cells. The current study focussed on trying to identify the stem/progenitor cell population(s) in the normal and (pre)malignant human cervical epithelium by using cervical tissue from fetal origin, as well as healthy and diseased adult tissue.

Stem cells

It is known that all adult tissues possess tissue specific stem cells which serve to replace worn out tissues [8]. These cells ensure the maintenance and proper functioning of the respective tissues throughout the lifetime of an organism. To this end they have adopted self-renewal capacity by asymmetric division [9]. An additional important aspect of stem cells is their close interaction with their micro environment, the so called stem cell niche [10]. Although stem cells in general share common features, stem cells of individual tissues show marked differences.

Characterization of stem cells in the context of tissue homeostasis is essential to our understanding of tumorigenesis. Markers for stem cells or progenitor can be divided into different subtypes, like markers of stem cell division and immortality (telomerase, Bcl-2, DNA label retention), signaling protein markers, cell surface antigens, transcription factors, detoxifying proteins as well as differentiation markers like nestin and cytokeratins [11]. Recently, progress has been made in understanding how epithelial stem cells develop into tissues such as skin, the corneal epithelium and the intestine [12-14].

Progenitor cells in uterine cervical epithelium

In human uterine cervical epithelium very few studies have so far been undertaken to identify progenitor or stem cell populations. A study on Ep-Cam, an epithelial adhesion molecule, showed that its expression in cervical squamous epithelium is associated with abnormal proliferation of cell populations that are not committed to terminal differentiation [15]. A study on the Wnt-signaling proteins Wnt 4,5a,7a in the mouse reproductive tract, and catenin-subtype immunostaining studies in cervical tissues, showed complex results, which are not readily applicable for studies in paraffin material [16-18].

In our studies we selected immunohistochemical markers applicable in paraffin material because of the importance to be able to properly judge the morphology of the tissue while interpreting the immunohistochemical results. For example as summarized in chapter 1, p63 has been described to be a suitable marker for epithelial tissue progenitor cells in several other epithelia including skin, esophagus and urothelia [19-21]. Bcl-2 is used as a marker for protection against apoptosis [22] while Ki-67 is a well defined marker for proliferation [23;24]. From the detoxifying enzyme group we choose to study gluthation S-transferase π [25]. Cytokeratins were used as differentiation markers to identify and characterize the different epithelial cell populations in our tissue preparations. Studies on other epithelia suggested the use of cytokeratin antibodies as progenitor cell markers in skin and eye [11;12]. We used a range of cytokeratins known as markers for basal cells including CK 5, 14, 17 and 19, as well as markers for glandular differentiation i.e. CK 7,8,18, and cytokeratin markers for squamous differentation, i.e. CK 13 [26].

The reserve cell

In our search for the progenitor or stem cells of cervical epithelium our special interest was drawn to the subcolumnar reserve cell population. In the literature the origin of reserve cells has long been a major subject of discussion in the first half of the 20th century [27-29].

At that time studies on uterine cervical carcinogenesis focussed on the regeneration process in the uterine cervical epithelium. This process of prosoplasia [30], later called squamous metaplasia, starts from reserve cell hyperplasia [31], and will normally result in normal mature squamous epithelium. The prominent role of the reserve cell in this physiological repair process suggests a progenitor role in cervical epithelium. We assumed that in fetal tissue a relatively large fraction of progenitor cells should be present. Therefore we investigated fetal human uteri at several stages of gestation and could in this way visualize the "birth" of the endocervical reserve cell (chapter 2) [32]. We conclude, on basis of their immunophenotype, that reserve cells are derived from Mullerian epithelium at midgestational age and have the same marker profile as adult reserve cells [32], hereby strongly supporting the progenitor cell nature of reserve cells. As gestational age increases a subpopulation of reserve cells, expressing cytokeratin 17, can be identified.

Progenitor cells should evidently be present in all samples of an adult epithelium, as they guarantee the regeneration capacity of the tissue. We indeed observed reserve cells in all adult cervices examined, along the entire cervical canal with concentrations of reserve cells at the squamo-columnar junction and high in the endocervical canal (chapter 4).
Our phenotyping studies of reserve cells showed that these cells express gluthation S-transeferase π, p63, bcl-2 and CK 5,7 and 8 [33;34]. Earlier studies revealed that these reserve cells show no or only very limited immunoreactivity for Ki-67 [23]. This means that they exhibit a basal cell type profile and are protected against apoptosis and toxifying influences, suggesting a progenitor/stem cell funtion in the cervical epithelium.

Based on their CK 17 expression profile two distinct reserve cell subpopulations can be defined. We propose, that a strongly CK 17 positive population close to the squamo-columnar has a progenitor cell function for both the squamous epithelium and the columnar cells in this region, while the subpopulation of reserve cells with a much weaker or absence of CK 17 expression, in the higher endocervix, is suggested to have a progenitor cell funtion for the endocervical columnar epithelium (chapter 4).

Cancer stem cells in cervical carcinoma

There is evidence that cancer is maintained by a subpopulation of cells termed cancer stem cells, which are responsible for tumour maintaince and for regrowth of cancer following conventional treatment [35;36]. At present little is known about the relationship between stem cells in normal for example stratified epithelia and stem cells in squamous cell carcinoma derived therefrom. A model proposed by Watt et al [37] suggests that during tumour development the pathways that control tissue homeostasis e.g. proliferation and apoptosis, are lost. There seems no evidence for expansion of the normal stem cell department.

For cervical (pre) malignancies only limited marker studies have so far been performed. A recent study by Ye et al [38] describes the expression of, amongst others, nanog a protein highly expressed in undifferentiated embryonic stem cells, in malignant cervical epithelial cells. They conclude that stem cell proteins have a role in cervical carcinogenesis and that they regulate the cell differentiation, proliferation and maintain cancer cell pluripotency. Expression levels of these constituents were higher in carcinoma than in CIN lesions, and higher in CIN than in normal epithelium. Expression of these markers in normal epithelium of the cervix was not indicated by these authors. Oct 3/4, a transcription factor, highly expressed in immature germ cell tumors was negative in all cases of normal cervix and the lesions derived therefrom [39;40].

The immunohistochemical markers we used in order to identify the progenitor cell of the human cervical epithelium showed also strong expression of reserve cell constituents in CIN lesions and in squamous cell carcinoma. In Chapter 5 we conclude that all different grades of CIN lesions and all squamous carcinomas show moderate cytoplasmic activity for Glutathion S-transferase π. In Chapter 3 we showed p63 expression in all grades of CIN. In low grade CIN lesions the staining was limited to the basal compartment, while in high grade CIN lesions the staining was noted throughout the full thickness of the epithelium. We also stated that p63 and CK 17 positive reserve cells could be the putative target cell for HPV infection. Regauer et al [41] conclude, based on our findings, that well-delineated fields of HPV-related intra epithelial neoplasia, may occur from these stem/reserve cell populations.

In Chapter 6 we describe that cytokeratins 8 and 17 can be used as a diagnostic tool for cervical carcinoma detection in cytologic samples. We could identify the CK 17 positive reserve cells [42], with the percentage of CK 17 positive cells being higher when the lesions were of higher dysplastic degree, suggesting a proliferation of reserve cells as dysplasia progresses. We therefore suggest that this marker can be regarded as a diagnostic and prognostic indicator.

Conclusion

As a general conclusion from these studies it can be stated that the origin of the reserve cell could be defined and that the compartment of the basal cytokeratin 17 positive cells of the cervical epithelium exhibits stem cell features. These reserve cells are positive for p63 and bcl-2 and negative for Ki-67. Based on immunohistochemical studies, a progenitor role for these reserve cells is proposed in the normal and carcinogenic development of the epithelial compartments in the uterine cervix.

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8

Summary

Summary

The current study was designed to give more insight in cervical carcinogenesis. It is well known that a persisting infection with high risk human papillomavirus (HPV) is a necessary condition in the carcinogenesis of cervical cancer. A lot of epithelial carcinoma originate from a progenitor or stem cell population of the epithelium. We therefore assumed a progenitor or stem cell model in the development of human uterine cervical cancer. By identifying the progenitor or stem cell in the uterine cervical epithelium we tried to find the putative target cell for high risk HPV infection. We used several immunohistochemical markers in paraffin material which enabled us to correlate morphology with immunohistochemical marker expression at single cell level. An important candidate for the progenitor cell of the cervical epithelium is the reserve cell.

In chapter 2 we studied fetal human tissue from different gestational ages by using p63, bcl-2, ki-67 and cytokeratins 5,7,8,13,17,18 and 19. We presumed high concentrations of progenitor cells in fetal human ontogenesis. In this study we tried to identify the progenitor/stem cell in human fetal uterine epithelium and studied the origin of the reserve cell. We concluded that reserve cells are at first identified at approximately 20 weeks of gestation. They are first noted under Müllerian type columnar cells lining the developing uterine cavity. There is considerable overlap in expression profiles of Müllerian cells and reserve cells for p63, bcl-2 and cytokeratins 5,8 and 18 at this stage of development, with increasing gestational age expression localizes to respective cell compartments. Eventually the phenotype of these cells correspond fully with that described for adult reserve cells and endocervical cells. These observations support the idea that reserve cells are the progenitor cells of the human cervical epithelium.

In chapter 3 we used p63 and cytokeratin 17 in normal cervical tissue and in precursor lesions. By applying these markers for basal cells we tried to identify the progenitor cell of the

uterine cervical epithelium. We noted p63 expression consistently in the nuclei of reserve cells, of hyperplastic reserve cells and of cells of the basal cell layer in the ectocervical epithelium. Cytokeratin 17 only stained endocervical reserve cells and reserve cell hyperplasia. From the combination of p63 positivity and CK 17 positivity in reserve cells we conclude that the reserve cell could be the progenitor cell of the epithelium.

In chapter 4 we studied the distribution pattern and immunoprofile of reserve cells along the entire length of the adult cervix by the use of p63, bcl-2, cytokeratins 5,7,8 and 17. We tried to identify reserve cell populations with specific keratin phenotypes being the progenitor cell population of the cervical epithelium.
We observed reserve cells along the entire cervical canal in all cervical samples independent of age of the patient. There were reserve cells in the vicinity of the transitional zone, followed by a discontinous pattern under the endocervical columnar cells. High concentrations of subcolumnar reserve cells were found proximal in the endocervical canal. The immunoprofile of reserve cells for p63, bcl-2, cytokeratins 5,7 and 8 was the same in all parts of the cervix. Cytokeratin 17 expression showed a diminishing gradient from distal to proximal in the cervical canal. This suggests a subpopulation of cytokeratin 17 negative cells that can be the progenitor cells of columnar epithelium. While the cytokeratin 17 positive reserve cell population could be the progenitor cell population for the endocervical and ectocervical epithelium.

In chapter 5 we used the detoxification enzyme Gluthation S-transferase π (GST π) in a well defined subset of normal cervical tissue, precursor lesions (CIN I,II and III) and carcinoma to try to predict progression or regression of the lesions. In literature there were conflicting results. In our study we found no differences in GST π expression in the precursor lesions. We also studied the expression profile in reserve cells. A progenitor cell should be protected against toxifying agents to be able to survive as long as possible. We concluded reserve cells and reserve cell hyperplasia to be strongly positive for GST π. In our opinion this finding supports the possible progenitor role of the reserve cell.

In chapter 6 we focussed on markers for progressive potential of premalignant lesions in cytologic smears. We used antibodies to cytokeratin 8 and 17 in order to identify dysplastic, progenitor cells. We tried to predict the behaviour of the lesions. From this study we concluded that the more cytokeratin 8 and 17 positivity, the more progressive potential.

Finally we conclude a central role for the reserve cell, as a the progenitor cell of the uterine cervical epithelium.

9

Samenvatting

Samenvatting

De studies die in dit proefschrift worden beschreven hebben als doel het inzicht in de ontwikkeling van baarmoederhalskanker te vergroten. Het is algemeen bekend dat een persisterende infectie met hoogrisico Humaan Papillomavirus (HPV) een noodzakelijke voorwaarde is voor de ontwikkeling van baarmoederhalskanker. Veel typen epitheliale kanker ontstaan vanuit een voorlopercel of stamcel populatie. Wij veronderstelden om die reden dat een stam/voorlopercel een rol speelt in de ontwikkeling van de baarmoederhals en baarmoederhalskanker. Door het identificeren van de voorlopercel van het epitheel van de baarmoederhals hebben wij geprobeerd om de doelwitcel te vinden voor een hoogrisico HPV infectie. Wij gebruikten diverse immunohistochemische markers in parafine materiaal. Dit gaf de mogelijkheid om morfologie te correleren aan immunohistochemische marker expressie op celniveau. Een belangrijke kandidaat voor de voorlopercel in de baarmoederhals is de reservecel.

In hoofdstuk 2 werd foetaal humaan baarmoederhalsweefsel gebruikt van oplopende amenorrhoe duur. Wij veronderstelden dat foetaal weefsel een ruime hoeveelheid voorlopercellen bevat en probeerden de oorsprong van de reservecellen vast te stellen. De weefsel coupes werden gekleurd met p63, bcl-2, ki67 en cytokeratines 5,7,8,13,17,18 en 19. Reservecellen werden voor het eerst gezien bij een amenorrhoeduur van 20 weken. Deze cellen werden gezien onder het Müllerse epitheel dat de zich ontwikkelende baarmoederholte bekleedt. Er werd een duidelijke overlap gezien in het expressie profiel van Müllers epitheel en foetale reservecellen voor p63, bcl-2 en cytokeratines 5,8 en 18. Met een toename van de amenorrhoeduur ontwikkelde het phenotype van de foetale reservecellen zich richting het expressiepatroon zoals dat in volwassen reservecellen wordt gezien. Uit deze bevindingen concludeerden wij dat reservecellen ontstaan uit Müllers epitheel en dat zij de voorlopercel voor het baarmoederhals epitheel zijn.

In hoofdstuk 3 richtten wij ons op de identificatie van de voorlopercel door het gebruik van basaalcelmarkers. Wij gebruikten p63, een homoloog van het tumorsuppressorgen p53 en cytokeratine 17 in normaal baarmoederhalsepitheel en premaligne afwijkingen. Wij stelden een consistent expressiepatroon vast voor p63 in de kernen van reservecellen, in reservecelhyperplasie en in de basale laag van het ectocervicale epitheel. Cytokeratine 17 kleurde reservecellen en reservecelhyperplasie. Wij concludeerden dat zowel p63 als cytokeratine 17 geschikte markers zijn voor het opsporen van de voorlopercel van het baarmoederhalsepitheel. Aangezien zowel p63 als cytokeratine 17 de reservecellen aankleurde stellen wij, dat deze cellen, de voorlopercelpopulatie vormen.

In hoofdstuk 4 bestudeerden wij het distributiepatroon en markerprofiel van reservecellen langs het hele epitheeloppervlak van de baarmoederhals. Wij maakten gebruik van p63, bcl-2, cytokeratine 5,7,8 en 17. Het doel van deze studie was om subpopulaties van reservecellen met specifieke keratine expressiepatronen op te sporen om voorlopercelpopulaties te kunnen vast stellen. In elke baarmoederhals zagen wij reservecellen, onafhankelijk van de leeftijd van de patiënt. De reservecellen toonden een specifiek distributiepatroon. Zij waren aanwezig in de buurt van de transformatiezone, vervolgens discontinue onder het endocervicale cylinder epitheel en uiteindelijk hoog in het cervicaal kanaal was weer een grote hoeveelheid reservecellen aanwezig. Het markerprofiel van de reservecellen was identiek voor p63,bcl-2, cytokeratine 5,7 en 8. Voor cytokeratine 17 zagen wij een afnemende expressie van distaal naar proximaal. Dit suggereert een subpopulatie van cytokeratine 17 negatieve reservecellen die de voorlopercelpopulatie voor het cylinderepitheel zou kunnen zijn.

In hoofdstuk 5 gebruikten wij het detoxificatie enzym Gluthation S-transferase π (GST π) in een reeks van normaal baarmoederhals epitheel, premaligne afwijkingen en carcinoom om de aard van de laesie te voorspellen. De in de literatuur beschreven resultaten zijn niet eenduidig. Wij stelden vast dat GST π alle premaligne afwijkingen op dezelfde manier aankleurden. In deze studie richten wij ons op het expressieprofiel van de reservecellen.

Wij vonden een sterke aankleuring van alle reservecellen. Naar onze mening ondersteunt dit de gedachte dat reservecellen de voorlopercellen van het baarmoederhals epitheel zijn. Een voorlopercel dient immers beschermd te zijn tegen invloeden van buiten af.

In hoofdstuk 6 hebben wij onderzoek gedaan naar markers die informatie geven over de kans op progressie van een premaligne laesie in cytologisch materiaal. In de veronderstelling dat reservecellen betrokken zijn in het dysplastisch proces hebben we antilichamen tegen cytokeratine 8 en cytokeratine 17 gebruikt om dysplastische cq reservecellen te kunnen identificeren. Op deze manier probeerden wij het gedrag van de laesies in te schatten. Uit deze studie concludeerden wij dat een toename van de immuunreactiviteit voor cytokeratine 8 en 17 gepaard gaat met een grotere kans op progressie van een afwijking.

In hoofdstuk 7 worden de belangrijkste resultaten bediscussieerd in relatie tot de literatuur met betrekking tot stamcelonderzoek. De belangrijkste conclusie is dat de reservecel een centrale rol heeft in de ontwikkeling baarmoederhalsepitheel.

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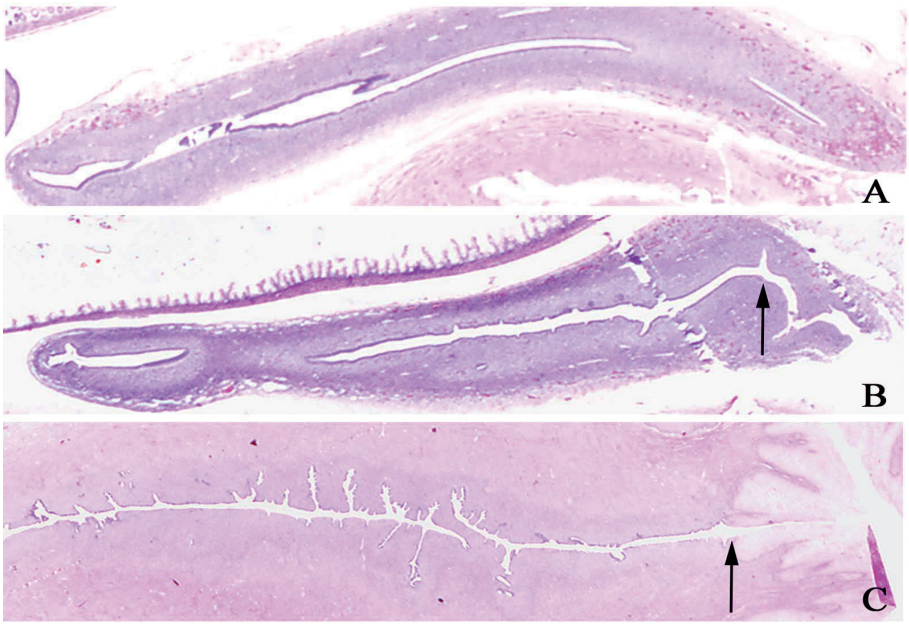
Color figures

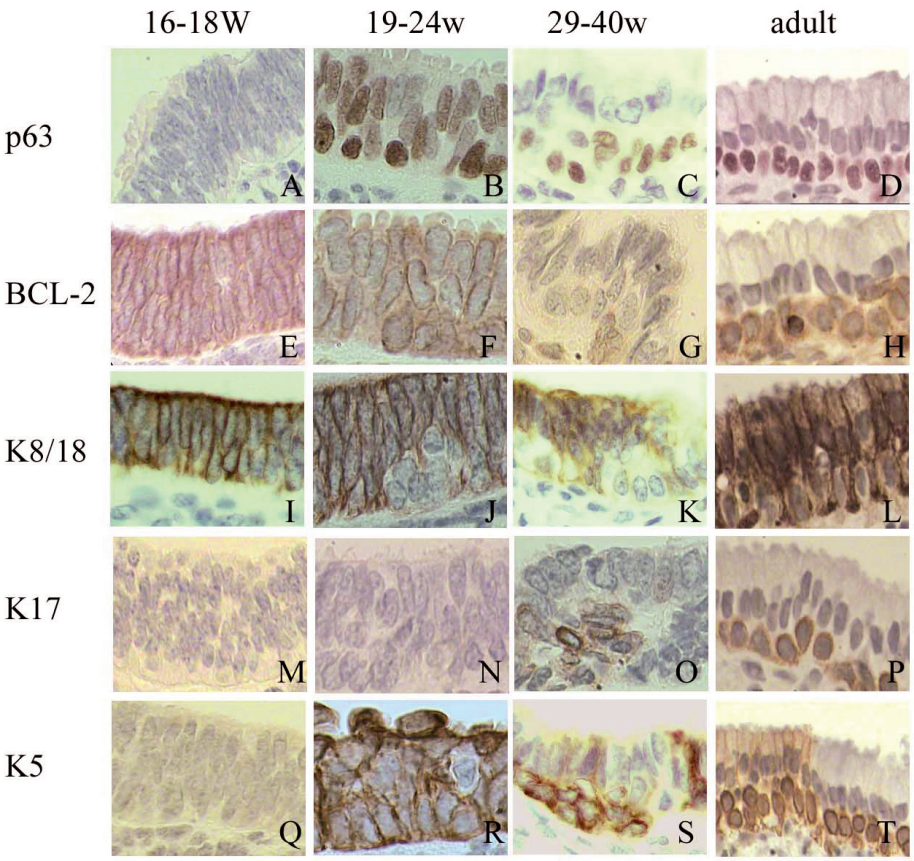
Chapter 2 - Figure 1



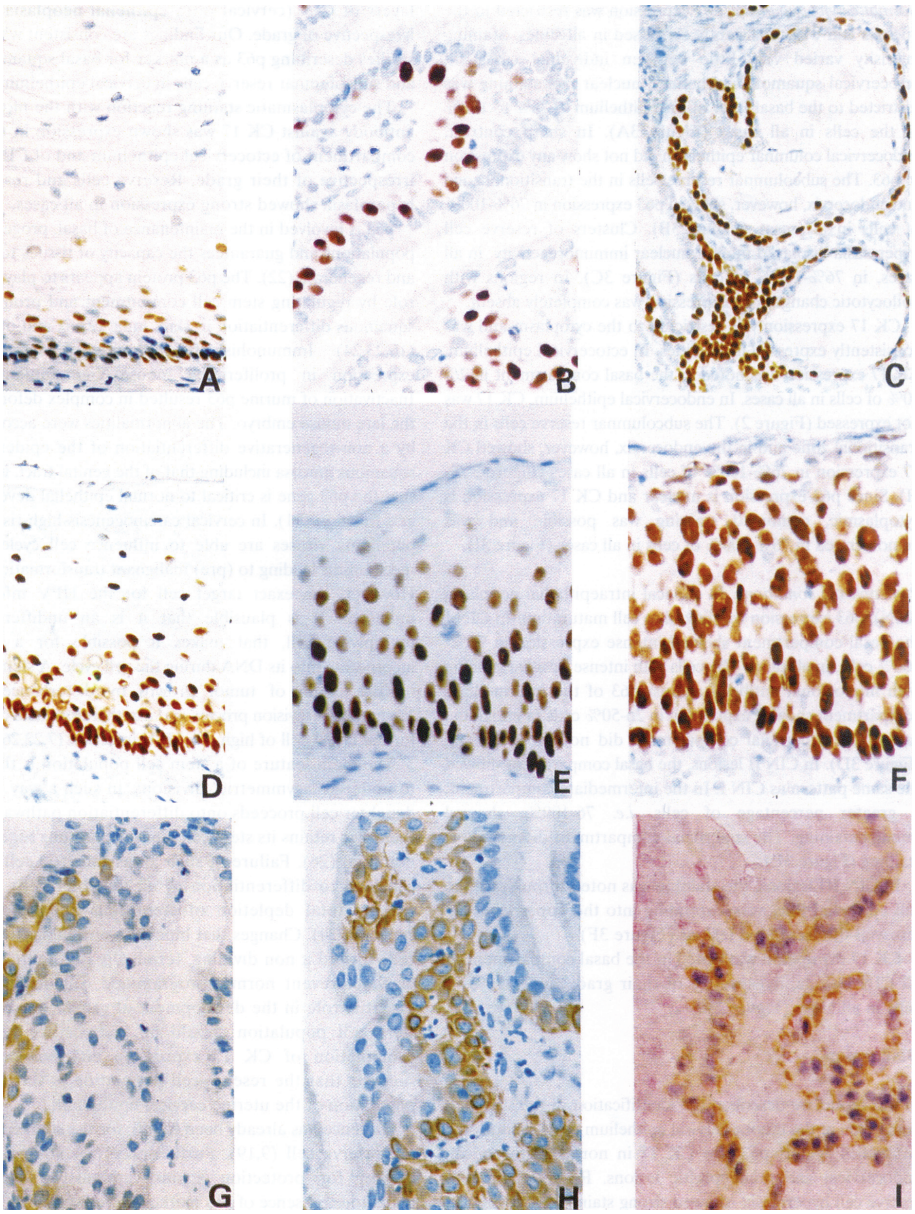
a.) Overview of the lower genital tract in a fetus gestational age 18 weeks. The primitive uterine cavity is present (left) and there is a solid cord of epithelial cells in the distal portion of the uterine cavity (right, Mullerian ducts). The transition between the uterine cavity and solid epithelial cord has yet to be established.

b.) Overview of lower genital tract in a 19 week old fetus. The transition between uterine cavity and solid epithelial cord has been established (arrow) The observed lumen is an artefact due to retraction of the solid epithelial cord from the underlying stroma. In the proximal portion of the primitive uterine cavity there is no endocervical differentiation.

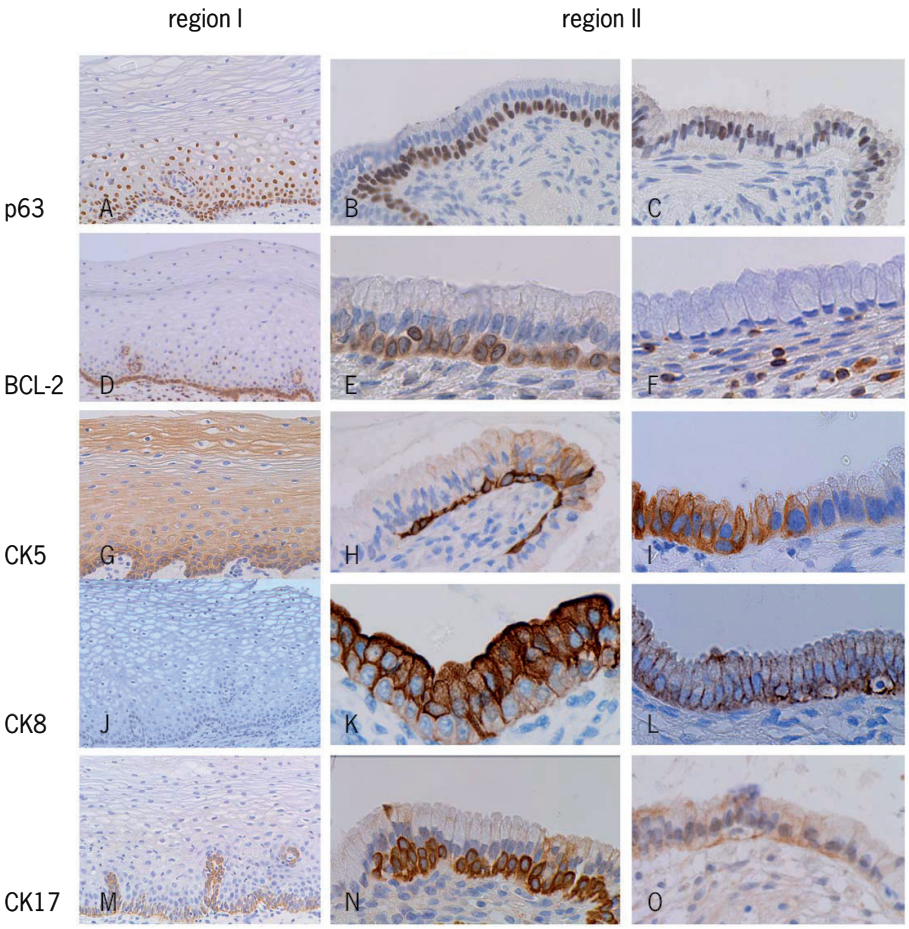
c.) Overview of the lower genital tract in a fetus gestational age 29 weeks. The arrow denotes the transition between the ectocervix, lined by non-keratinising squamous epithelium and the newly developed endocervix. This is very short (less than 1mm) and lined by endocervical and Mullerian type columnar cells.



The development of reserve cells in the human fetal uterine cervix from 16-18 weeks, 19-24 weeks, 29-40 weeks of gestation to adult and the development of the expression pattern of p63, bcl-2, cytokeratins 8,18,17 and 5 in the reserve cells and the overlying columnar epithelium.

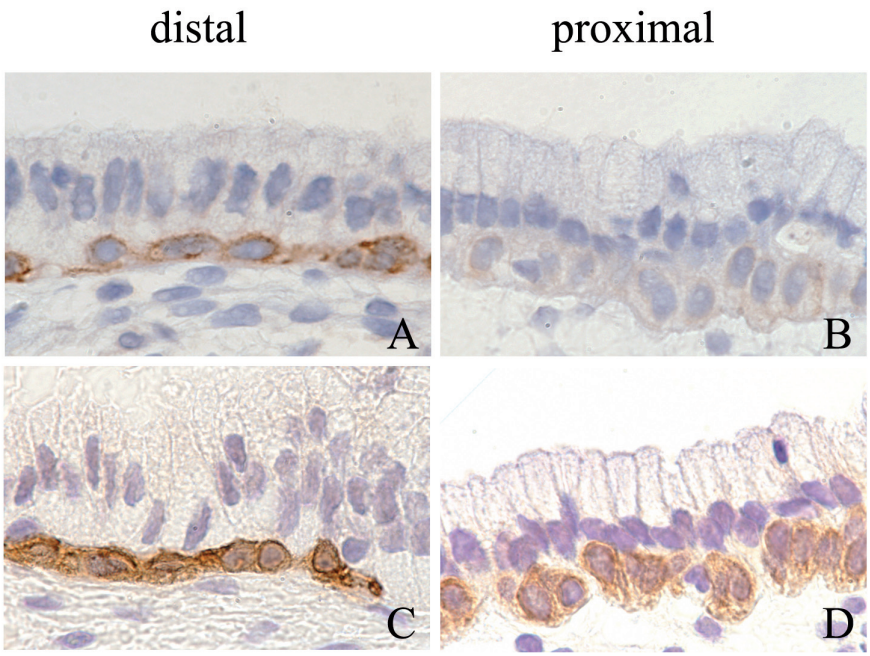


P63 expression in normal ectocervical epithelium (A), endocervical epithelium and reserve cell (B), reserve cell hyperplasia (C), in CIN I (D), CIN II (E), CIN III (F). Cytokeratin 17 expression in endocervical epithelium and reserve cells (G), reserve cell hyperplasia (H) and double staining of p63 and CK 17 (I).



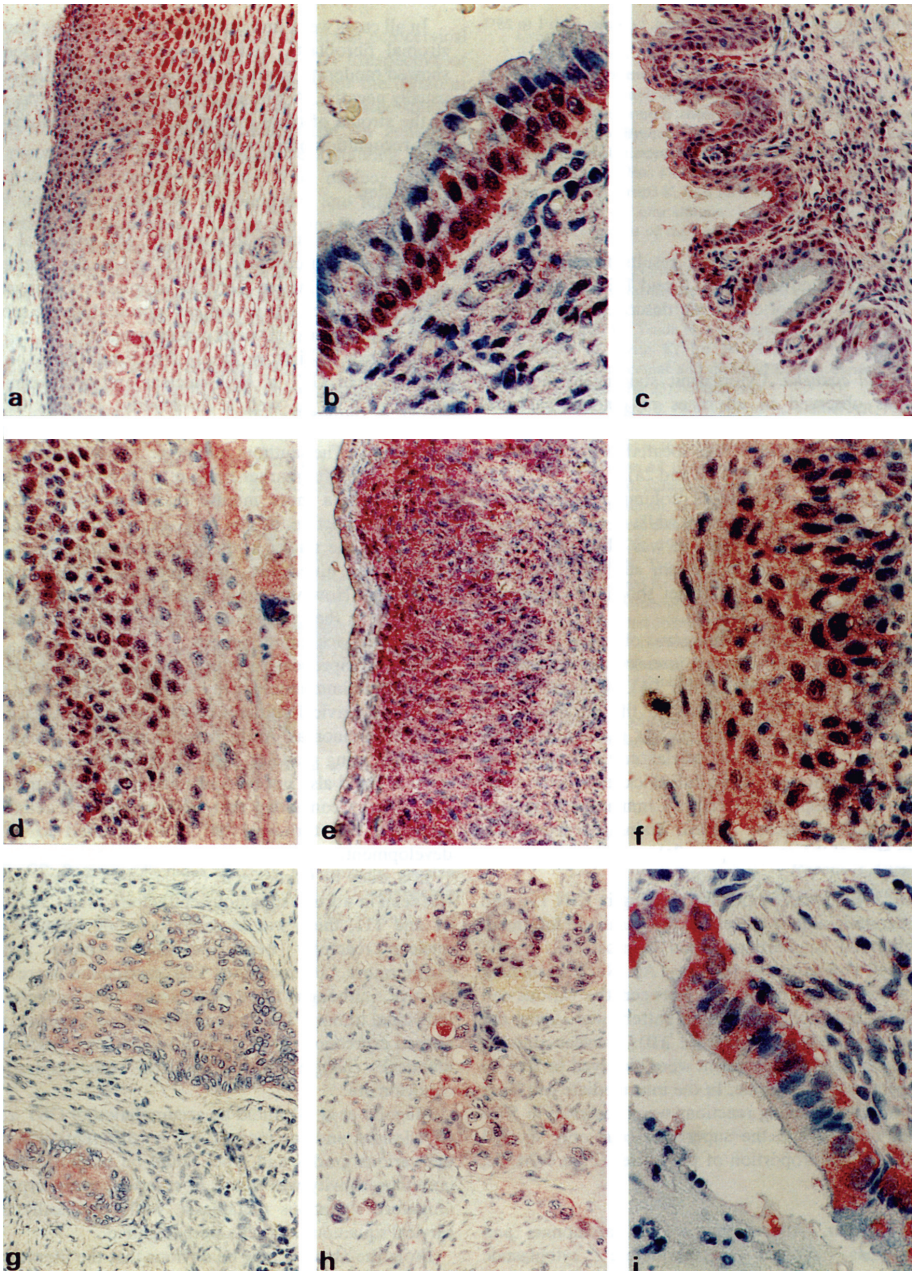
Immunostaining patterns for p63 (A-C), Bcl-2 (D-F), CK5 (G-I), CK8 (J-L) and CK 17 (M-O) of the ectocervical squamous epithelium (A,D,G,J,M), distally located columnar epithelium with underlying reserve cells (B,E,H,K,N) and more proximally located glandular epithelium without underlying reserve cells (C,F,I,L,O).

Chapter 4 - Figure 4



CK 17 expression patterns of distally (A,C) located subcolumnar reserve cells, close to the squamocolumnar junction, and (B,D) proximally located subcolumnar reserve cells in the upper third of the endocervix. Two different immunostaining protocols were applied, i.e. the automated staining procedure (A,B) and a staining protocol with optimized antigen retrieval (C,D).

Chapter 5 - Figure 2



GST π immunostaining reactivity in ectocervical epithelium (a), endocervical epithelium and reserve cells (b), immature squamous metaplasia (c), CIN I (d), CIN II (e), CIN III (f), squamous cell carcinoma (g), adenocarcinoma (h) and tubal metaplasia (i).

Dankwoord

Dankwoord

Promoveren kun je niet alleen. Graag wil ik gebruik maken van de gelegenheid om allen te bedanken door wier medewerking dit proefschrift tot stand kon komen. In de periode dat de studies van dit proefschrift zijn gedaan hebben velen vanuit allerlei windstreken hun bijdrage aan dit proefschrift geleverd. De steun en bereidheid van jullie allen om mij te helpen dit onderzoek tot een goed einde te brengen waardeer ik zeer.

Byzondere dank gaat uit naar de promotoren en copromotoren voor hun jarenlange steun en vertrouwen in de goede afloop: Prof dr ThJM Helmerhorst, Prof dr FCS Ramaekers, dr AHN Hopman, dr FMM Smedts.

Veel dank ben ik verschuldigd aan de leden van de Kleine Commissie: Prof dr CW Burger, Prof dr CJLM Meijer, Prof dr JW Oosterhuis. Ik dank hen voor het kritisch lezen van het manuscript en de opbouwende opmerkingen naar aanleiding daarvan.

De leden van de Grote Commissie wil ik bedanken voor hun bereidheid te oponeren: Prof dr JW Arends, Prof dr JWW Coebergh, Prof dr E Dzierzak, Prof dr EJ Kuijpers, Prof dr GP Vooijs.

Alle co-auteurs wil ik hartelijk bedanken voor hun bijdrage aan de studies en daarmee aan dit proefschrift: Jan Willem Arends, Janny Baars, Bert de Boer, Bram ter Harmsel, Theo Helmerhorst, Marjon Holterheus, Ton Hopman, Marie Jose Kok, Paul van der Linden, Ruud van Muyden, Diane Ploeger, Frans Ramaekers, Coen Schoots, Frank Smedts en Peter Vooijs.

De medewerkers van de laboratoria in Delft, Deventer, Maastricht en Winschoten. Hun werk was onmisbaar!

Secretaresses Bea Bral, Yolanda Verhaegen, Diane van den Bos-Monteny, Ingrid van den Bos, Chrisa van Dijk, Els Kruit en Marja van der Meer-Scheijgrond, dank voor de logistieke en administratieve ondersteuning.

Bibliothecaris van het Maasstad ziekenhuis Jacco van Meel en de bibliothecaresse van het Deventer ziekenhuis Marianne Boerstoel, veel dank voor jullie uitstekende literatuurservice.

Mattie Simons wil ik hartelijk bedanken voor de digitale ondersteuning en het maken van de figuren.

Paranimfen: Astrid Baalbergen, in de Daniel den Hoed kliniek begon onze samenwerking. Onze gemeenschappelijk interesse in de oncologie, snelle auto's en het moederschap op latere leeftijd vormen de basis voor onze vriendschap. Nu jij nog?!

Lieve Ruud, dank voor je liefdevolle ondersteuning in alle facetten van ons leven!

Curriculum Vitae

Jolise Martens werd geboren op 23 juli 1965 te Haarlem. Zij behaalde in 1983 haar eindexamen VWO-B aan het Peter Stuyvesant college te Curaçao. In dat zelfde jaar startte zij haar studie informatica aan de Rijksuniversiteit Leiden. Na het halen van het propedeutisch examen besloot zij in 1985 de overstap naar de studie geneeskunde te maken. In 1989 slaagde zij voor haar doctoraal examen en in 1992 voor het artsexamen. Na een periode van anio-schap gynaecologie in het IJsselland ziekenhuis te Capelle aan de IJssel en het Reinier de Graaf gasthuis te Delft begon zij in 1995 aan de opleiding tot gynaecoloog in het Rotterdams opleidingscluster.

Het eerste jaar opleidingsjaar werd in het Reinier de Graaf gasthuis (opleider dr J.C. Kuijpers), gevolgd, haar academische periode 1996-1999 in het Erasmus MC te Rotterdam (opleiders: Prof dr H. Wallenburg en Prof dr Th.J.M. Helmerhorst). Zij vervolgde haar opleiding in het Zuiderziekenhuis (opleider dr M. van Lent) en heeft zij haar opleiding afgesloten met een differentiatie jaar oncologie in de Daniël den Hoed kliniek te Rotterdam. Haar opleiding tot gynaecoloog werd op 1 maart 2001 afgerond.

Vervolgens begon zij haar werkzame leven als gynaecoloog, met taakstelling oncologie, in het Deventer Ziekenhuis. Om de oncologische vaardigheden verder te ontwikkelen werd een nulaanstelling in het AMC verkregen. Dit gaf de mogelijkheid om deel te nemen aan multidisciplinaire oncologische besprekingen. Tevens kreeg zij de gelegenheid haar operatieve vaardigheden te vergroten. Vanaf 1 november 2007 werd haar carrière als gynaecoloog voortgezet in het Maasstadziekenhuis. De overgang van Deventer naar Rotterdam geeft haar de mogelijkheid het oncologisch deelgebied verder te ontwikkelen en uit te diepen.

De basis voor het onderhavig onderzoek werd als anio in Delft gelegd. Geïnspireerd door de zeer enthousiaste patholoog en huidige copromotor, dr F. Smedts. Gedurende de opleiding bleef de wetenschap trekken en werd de in Delft ingezette lijn voortgezet. De samenwerking met de afdeling moleculaire celbiologie te Maastricht in de persoon van Prof dr F.C.S. Ramaekers en dr A. Hopman werd een feit. Het fulltime werk in de perifere praktijk, in combinatie met het moederschap, heeft er toe geleid dat dit promotie onderzoek pas na 10 jaar wordt afgerond.
Naast haar klinische werkzaamheden was zij steeds bestuurlijk actief.

Jolise is gehuwd, met mij, Ruud van Muyden en samen hebben wij twee zonen, Sweder (2004) en Witte (2006).