Surrogate Endpoint Biomarkers and Their Modulation in Cervical Chemoprevention Trials

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BACKGROUND. Surrogate endpoint biomarkers (SEBs) are used as intermediate indicators of a reduction in cancer incidence in chemoprevention studies. SEBs should be expressed differentially in normal and high risk tissue; appear at a well defined stage of carcinogenesis; be studied with reasonable sensitivity, specificity, and accuracy; and be modulated in chemoprevention trials. The concept of SEBs may be useful in the trials of many new therapies.

METHODS. The current review includes a comprehensive review of the literature. Many SEBs have been the subject of intense study and include quantitative histopathology and cytology, proliferation markers, regulation markers, differentiation markers, general genomic instability markers, and tissue maintenance markers. Because of the critical biologic and epidemiologic role of the human papillomavirus (HPV) in cervical carcinogenesis, the relation between these markers and HPV should be considered. In addition, biomarkers of HPV infection and its regression should be sought.

RESULTS. Several chemoprevention trials have been published that have included the use of SEBs. The biomarkers that appear most promising in these clinical trials can be measured quantitatively and reproducibly: quantitative histology and cytology, proliferating cell nuclear antigen (PCNA), MIB-1, MPM-2, HPV viral load, epidermal growth factor receptor, polyamines, and ploidy. The markers that have been demonstrated to be modulated in chemoprevention trials in the literature are quantitative histology and cytology, PCNA, MPM-2, HPV viral load, and polyamines.

CONCLUSIONS. The surrogate endpoint biomarkers of most interest in future research should correlate well with HPV infection, be modulated by several therapeutic agents, and have limited variability and ease in measurement. ***Cancer*** **2001; 91:1758–76.** *© 2001 American Cancer Society.*

KEYWORDS: surrogate endpoint biomarkers, cervix, chemoprevention, squamous intraepithelial lesions.

Cervical lesions have long been believed by pathologists to be the best model with which to study the progression from mildly dysplastic lesions to severely dysplastic lesions to invasive carcinoma. The accessibility of the cervix affords clinicians the ability to observe cervical lesions over time with the magnifying lens of the colposcope; these lesions show progressive vascular atypia as they advance to neoplasms. Its accessibility also allows the cervix to be easily sampled cytologically with the Papanicolaou (Pap) smear and histologically with the colposcopically directed biopsy. Just as the biopsy shows predictable changes as lesions progress toward invasion, the Pap smear provides a cytologic model for the progression of disease. These factors make the cervix a unique organ that is well suited to the development of screening and diagnostic interventions.

Chemoprevention Trials

Chemoprevention refers to the use of chemical agents (micronutrients, pharmaceuticals) to prevent or delay the development of cancer in healthy populations or patients with precancerous tissue changes.[1,2] These agents, which block the initiating and promoting events of carcinogenesis, are a novel strategy fueled by the philosophy that cancer is a multistep process preceded by identifiable precursors. In an attempt to prepare the oncologic community for this paradigm shift, Sporn cautioned that intervention in the preneoplastic phase is necessary because carcinogenesis, not invasive or symptomatic cancer, in the actual disease process. Metastatic and invasive disease must be considered to be clinical and pathologic end stages, by which time it may not be possible to prevent further disease progression.[3]

Chemoprevention offers the possibility of learning a great deal regarding the carcinogenic process by reversing it. To be suitable for chemoprevention, medications should be well tolerated and have low toxicity. To our knowledge several chemoprevention trials have been performed in patients with cervical intraepithelial neoplasia (CIN). Micronutrients, retinoids, and polyamine synthesis inhibitors have been used.[1] SEBs specifically modulated by chemopreventive agents allow endpoints of a shorter duration than the development of cancer and provide a window into cancer biology.[4,5]

Surrogate Endpoint Biomarkers

SEBs are used as intermediate indicators of cancer incidence reduction in chemoprevention studies. Before SEBs are deemed useful in chemoprevention trials, several criteria must be met. These are well outlined by Kelloff et al.: the SEB must be expressed differentially in normal and high-risk tissue; the marker should appear at a well defined stage of carcinogenesis; the marker and its assay must provide acceptable sensitivity, specificity, and accuracy; the marker should be easily measured; the marker should be modulated by chemopreventive agents; and, finally, modulation of the SEB should correlate with a decrease in the cancer incidence rate.[4] Several types of SEBs already are in use in clinical trials. A complete list of potential SEBs for the cervix is presented in Table 1.[6–8]

Because HPV is critically important in the carcinogenesis of cervical carcinoma, SEBs must be studied in relation to HPV. zur Hausen and de Villiers have written an important review on the subject.[9,10] Specific viral transforming genes E6 and E7 from HPV types 16 and 18 act as oncogenes; their expression emerges as necessary, but not sufficient, for malignant conversion. There does not appear to be a consistent locus in the host for viral integration; however, there is a striking pattern of integration with respect to the viral genome. Integration frequently disrupts E1 and E2 open reading frames; disrupting these regulatory genes and their regulatory proteins annuls regulation of gene expression. E6 shares functional and structural features of SV40 large T antigen and adenovirus 5 E1B and in vitro promotes degradation of p53 via the ubiquitin-dependent protease system. E7 shares functional and structural features of adenovirus E1A and can complex with pRb. E6 and E7 have been found to stimulate cell proliferation and are responsible for the

TABLE 1
Classes of Biomarkers in the Cervical Epithelium

Quantitative histopathologic and cytologic markers
Nuclei (abnormal size, shape, texture, pleomorphism)
Nucleoli (abnormal number, size, shape, position, pleomorphism)
Nuclear matrix (tissue architecture)
Proliferation markers
Proliferating cell nuclear antigen
Ki-67, MIB-1
Labeling indices (thymidine, BrdU)
Mitotic frequency (MPM-2)
Regulation markers
Tumor suppressors (p53, Rb)
HPV viral load and oncoprotein expression
Oncogenes (ras, myc, c-erb B-2)
Altered growth factors and receptors (epidermal growth factor receptor, transforming growth factor-β, cyclin-dependent kinases, retinoic acid receptors)
Polyamines (ornithine decarboxylase, arginine, ornithine, putrescine, spermine, spermidine)
Arachidonic acid
Differentiation markers
Fibrillar proteins (cytokeratins, involucrin, cornifin, filaggrin, actin microfilaments, microtubules)
Adhesion molecules (cell-cell: gap junctions, desmosomes) (cell-substrate: integrins, cadherins, laminins, fibronectin, proteoglycans, collagen)
Glycoconjugates (lectins, lactoferrin, mucins, blood group substances, glycolipids, CD44)
General genomic instability markers
Chromosome aberrations (AgNORs, micronuclei, three-group metaphases, double minutes, deletions, insertions, translocations, inversions, isochromosomes, FHIT)
DNA abnormalities (DNA hypomethylation, LOH, point mutations, gene amplification)
Aneuploidy (measured by flow cytometry)
Tissue maintenance markers
Metalloproteinases
Telomerases
Apoptosis and antiapoptotic markers

BrdU: bromodeoxyuridine; HPV: human papillomavirus; AgNORs: silver-stained nucleolar organizer region protein; FHIT: fragile histidine triad; LOH: loss of heterozygosity.
Modified from Mitchell MF, Hittelman WK, Lotan R, Nishioka K, Tortolero-Luna G, Richards-Kortum R, et al. Chemoprevention trials and surrogate end point biomarkers in the cervix. *Cancer* 1995;76(10 Suppl):1956–77.

genetic instability of the infected cell. The transforming gene's transcriptional and functional activity is regulated by the host cell genome. Mutational modifications of the host cell genome appear to be required for progression to invasion.[9,10]

QUANTITATIVE CYTOLOGIC AND HISTOPATHOLOGIC MARKERS

Nuclear Features, Nucleolar Features, and Tissue Architecture

Cytologic and histopathologic markers include nuclear features, nucleolar features, and tissue architecture. Nuclear features of interest include grade, shape, area, optical density, texture, nuclear pleomorphism, and ploidy (as estimated by DNA content). Nucleolar features of interest include size, shape, and position. Tissue architectural measurements exploit the finding that disordered nuclei are crowded and irregular. These markers now are being measured quantitatively using stoichiometric stains viewed by computer-assisted video imaging devices. Quantitative cytology and histopathology enable an objective, repeatable measure of what is seen by the pathologist.[11,12]

Cytologic and histopathologic diagnosis is the gold standard for the diagnosis of cancer. The pathologist judges cells to be dysplastic and cancerous based on predictable changes in nuclear shape, size, and grade, as well as in the relation of the cells to each other. Stoichiometric stains and refined cameras offer the possibility of obtaining a numeric value for DNA content, texture changes, and pleomorphism. Tissue architecture as measured by nuclear mapping allows the relation between cells to be studied quantitatively. Because these objective, repeatable measurements provide quantitative assessments of the cytopathologic diagnosis, they may be the best of all SEBs.[13–15]

To our knowledge the majority of the work presented to date in regard to the cervix has involved nuclear measurements of DNA content and texture.[16,17] To our knowledge no studies of nucleoli or tissue architecture have been published to date.

One of the best predictors of progression to invasion and decreased survival in most organ sites is aneuploidy.[18–20] Quantitative cytology and histopathology measure ploidy by estimating DNA content. Statistically significant increases in aneuploidy with disease progression are observed in both cytologic smears and histopathologic sections from the cervix. Nuclear area has been shown to increase in a predictable way. Measures of nuclear texture, reflecting chromatin stippling, also increase predictably as diagnoses progress from negative to atypia, low-grade squamous intraepithelial lesion (LGSIL), high-grade squamous intraepithelial lesion (HGSIL), and carcinoma. Combinations of DNA content and multiple texture features can be used to derive discriminate function scores for cytologic and histopathologic specimens that achieve high performance in discriminating diagnostic categories as measured by receiver operating characteristic curve analyses.[14] These algorithms can be used to predict which lesions are more likely to be associated with invasive tumors and which lesions may be more likely to progress to invasive carcinoma.[18–20]

To our knowledge the relation between HPV and quantitative cytology and histopathology has yet to be well studied. Because quantitative assays for HPV currently are available, the possibility of studying chromatin changes and HPV as tissues progress from normal to infected with HPV to SIL to carcinoma will be feasible.

DNA content has been demonstrated to be modulated in two separate analyses of tissue from a chemoprevention trial of α-difluoromethylornithine (DFMO) in patients with CIN type 3 (CIN-3).[21,22] In both studies, DNA content decreased with medication in the majority of patients; decreases were more significant in patients with a histologic response. Additional analyses of these same specimens revealed statistically significant changes in nuclear texture, indicating less clumping of chromatin with treatment.[23]

Quantitative cytologic and histopathologic measurement of DNA content and texture meet all the criteria of Kelloff et al. for good SEBs in the cervix and in other tissues; these markers are expressed differentially in normal and high-risk tissues; appear at a well defined stage of carcinogenesis; provide acceptable sensitivity, specificity, and accuracy; are easily measured; have been shown to be modulated by chemopreventive agents; and, finally, have been correlated with cancer incidence rates and survival.[4] It will be interesting to follow the studies of nucleoli and tissue architecture in the cervix as they unfold.

PROLIFERATION MARKERS

The rationale for the use of proliferation markers as SEBs is that cells with high proliferative activity are more likely to be associated with premalignant and malignant tissues. Proliferation is believed to be an early marker of disordered growth. It is hypothesized that increased proliferation is associated with more advanced lesions and that the distribution of proliferating cells in tissue may be indicative of the regulatory mechanisms that become dysfunctional during the multistep process. Proliferation is another biomarker that appears to have validity in many organ sites.[24,25]

Proliferation has been studied in cervical tissue with Ki-67 in frozen sections and with MIB-1 (an an-

tibody to Ki-67) and proliferating cell nuclear antigen (PCNA) in archival specimens. The distribution of proliferation by layer (basal vs. parabasal vs. superficial) might indicate growth-regulatory mechanisms; thus the relation between proliferation and growth dysregulation is of interest.[24,25]

PCNA

PCNA is a nuclear protein whose expression is associated with the late G_1-phase, S-phase, and early G_2 phase of the cell cycle.[26] An auxiliary protein to DNA polymerase, PCNA plays a critical role in initiating cellular proliferation. Studies of PCNA in cervical specimens have demonstrated increased activity as the lesions progress to invasion.[27–29] In studies of PCNA in invasive cervical carcinoma specimens from patients treated with retinoids and interferon, Ahn et al. found that PCNA was highly expressed in these lesions and decreased with tumor regression.[30]

To our knowledge studies of the relation between PCNA and HPV have not been performed to date. Such studies will be of great interest and may provide important information regarding viral integration and control of the cell cycle.

PCNA was modulated in a chemoprevention trial using DFMO. Statistically significant decreases in proliferation were noted in patients after 30 days of treatment. Decreases were more dramatic in patients with a histologic response than in nonresponders. Because DFMO is an antiproliferative, PCNA may be a biomarker that is well suited for studying response to this medication.[31]

PCNA meets the criteria for a good biomarker. It has been demonstrated to be expressed differentially in normal and high-risk tissue and is modulated by chemoprevention agents. However, to our knowledge extensive studies of its sensitivity, specificity, and accuracy have not been performed to date. With facilities for immunohistochemical analysis, this marker can be easily measured. Software exists for the quantitative analysis of PCNA.

PCNA appears to be a promising measure of proliferative activity in the cervix. The correlation of proliferation with regulatory and differentiation markers will be important for understanding the neoplastic process.

Ki-67 and MIB-1

Ki-67 is an antibody that was generated when mice were immunized with nuclear extract from the Hodgkin-derived L248 cell line.[24,25] Ki-67 immunolabeling correlates well with the cell cycle and is a widely employed marker of cell proliferation.

Konishi et al. studied the presence of Ki-67 in samples from normal, dysplastic, and cancerous cervices. Ki-67 was not measured quantitatively, but increases were noted during the normal menstrual cycle and as lesions progressed to carcinoma.[32] This study demonstrates that studies of proliferation markers need to control for patient age and the point in the menstrual cycle at which specimens are obtained. Ki-67 was measured quantitatively by image analysis by Devictor et al. and was found to show a statistically significant increase as the lesions progressed from CIN type 1 (CIN-1) to microinvasive carcinoma; no relation with HPV was noted.[33]

al-Saleh et al. studied the distribution of Ki-67 in the normal and dysplastic cervix and identified higher labeling indices in cervices infected with HPV types 16 or 18 and types 31, 33, or 35 compared with those infected with HPV types 6 or 11.[34] These results were confirmed further by the studies of Tervahauta et al.[35]

Ki-67 appears to be a good biomarker because it is expressed differentially in normal and high-risk tissue, markedly increases in high-grade lesions, and can be easily measured on frozen sections in the pathology laboratory. MIB-1, an antibody that detects Ki-67 in paraffin-embedded tissues, has been shown to have results similar to those noted with Ki-67. The increased ease of using this marker in paraffin-embedded tissue makes it an even better biomarker. However, to our knowledge no large studies of sensitivity, specificity, and accuracy have been undertaken to date, nor has Ki-67 or MIB-1 yet been shown to be modulated in a chemoprevention trial in the cervix.

Labeling Indices

Tritium has been used as a label for high-resolution radioautography and, when combined with thymidine, selectively labels nuclei that are synthesizing DNA. It is stable in the nucleus after incorporation and produces relatively little disturbance in the mitotic cycle. In a study by Richart,[36] CIN lesions were stained with tritiated thymidine and measured radioautographically. There were six levels of pathologic severity ranging from normal to carcinoma in situ (CIS). A linear relation between log of labeling index and severity of lesion was demonstrated. The calculation of doubling time for normal cervical tissue was 5.7 days, whereas that for CIS was estimated to be 11.3 hours.[36]

Bromodeoxyuridine (BrdU) is a thymidine analogue that also is incorporated into nuclear DNA. A monoclonal antibody identifying BrdU-containing nuclei was used in an immunohistochemical study of cellular proliferation. Fukuda et al.[37] used this antibody to study S-phase labeling in cone biopsy specimens. The levels of BrdU positive cells were 5.1% in normal epithelium, 12.3% in slight to moderate dys-

plasia, and 21.2% in severe dysplasia and CIS. In normal tissues, the majority of BrdU-staining cells were confined to the parabasal layer, whereas increases through the intermediate layer extending to full-thickness involvement were observed as lesions progressed to CIS.[37]

Although both tritiated thymidine and BrdU are expressed differentially in normal and high-risk tissue and appear at a well defined stage of carcinogenesis, both markers require exceptional facilities for measurement and thus fail the criterion that biomarkers should be studied easily.

Mitotic Frequency (MPM-2)

MPM-2, a mitotic monoclonal antibody, recognizes a phosphorylated epitope on a group of proteins that are phosphorylated at mitosis. Because it preferentially decorates cells in mitosis, the relation between mitosis and histopathologic change can be examined using this antibody. In a feasibility study using 23 cervical cone biopsies, Hu et al.[31,38] studied mitotic figure frequency per unit epithelium. There were statistically significant increases in MPM-2 staining in progressively severe lesions from normal cervix to CIN-1, CIN-2, and CIN-3 to invasive carcinoma; these quantitative measurements were obtained using computer-assisted image analysis. Moreover, the relative distance between the mitotic cells and the basal layer increased with the severity of the lesion, from CIN-1 to invasive carcinoma. Mitotic cells in high-grade lesions were distributed across the full thickness of the epithelium. These results suggest that proliferation becomes sequentially dysregulated both numerically and spatially during cervical carcinogenesis.[31]

To our knowledge the relation between MPM-2 and HPV has not been studied. Mitosis in HPV-infected lesions has been studied extensively; HPV induces mitosis. Because MPM-2 can be measured quantitatively using image analysis, correlation with HPV would be of interest.

MPM-2 was shown to be correlated with proliferative activity in the chemoprevention trial using DFMO. Decreased mitoses were noted in lesions with decreased proliferation, demonstrating that MPM-2 can be modulated by chemopreventive agents.[31]

Mitosis occurs differentially in normal and high-risk tissue and appears at a well defined stage of carcinogenesis. Although to our knowledge MPM-2 has not yet been studied extensively with regard to its sensitivity, specificity, and accuracy, the marker is easily measured. Because it has been shown to be modulated by a chemopreventive agent in the cervix, it appears to be a promising biomarker.[25,31]

REGULATION MARKERS

Regulation markers include tumor suppressors, HPV viral load and oncoproteins, oncogenes, growth factors and their receptors, polyamines, and arachidonic acid. These agents in their normal states help regulate cell growth. Their measurement may provide clues to the process of carcinogenesis.

Tumor Suppressors (p53, Rb)

The mutated or aberrantly expressed p53 tumor suppressor gene is believed to be responsible for $>$ 50% of human tumors. Cervical carcinoma is an exception. HPV E6 has been shown to functionally repress the action of wild-type p53.[9,10,39] Studies of cervical carcinoma show that the majority of cervical tumors (95%) are HPV positive and do not overexpress p53.[10] Similar findings exist for CIN lesions.[40,41] Those tumors that are HPV negative are more likely to have p53 mutations, but not exclusively so.[42–44] p53 mutations can be observed in metastatic lesions, suggesting a later role for full p53 alterations.

p53 is polymorphic at amino acid 72 of the protein, containing either a proline or arginine residue at this position. Storey et al.[45] reported an increased susceptibility for HPV-induced tumors in patients who are homozygous for the arginine residue. They showed a striking sevenfold increased risk for the development of cervical carcinoma.[45,46] This finding had great promise to explain the increased incidence and mortality of cervical carcinoma by the genetic susceptibility noted among some ethnic populations. However, three large cohorts of patients with SIL and carcinoma were found to not have the arginine-arginine mutation.[47–49]

The Rb gene is a tumor suppressor that is inherited in a dominant fashion, whereas the disease is recessive in nature. The active form of Rb is believed to be a dephosphorylated form of the protein that accumulates in the cell in the G_0/G_1-phase of the cycle. HPV E7 has been shown to functionally repress the action of Rb. If regulation of the cell cycle can be negated by HPV E7, then mutations of Rb may not be necessary in cervical carcinoma. Heck et al. and Scheffner et al. reported no macroscopic Rb-1 abnormalities or 13q allelic loss regardless of the HPV status in 28 cases of invasive cervical carcinoma.[50,51]

Because of the mechanism of cellular transformation in HPV-infected lesions and the small number of HPV negative SILs and carcinomas, neither p53 nor Rb is considered to be a good biomarker in the cervix.

HPV Viral Load and Oncoprotein Expression

HPV can be measured quantitatively using polymerase chain reaction (PCR) quantification of HPV types 16

and 18 E7 mRNAs.[52] Viral load has been correlated with severity of disease, with a higher viral load corresponding to higher grade lesions.[52,53] Viral load also has been demonstrated to correlate with disease persistence in a cohort of patients enrolled in a chemoprevention study. The relation between the compound under study, β-carotene, and the viral load was not reported.[52]

The measurement of HPV viral load is a promising biomarker. To our knowledge little is known to date regarding the relation between viral load and disease progression, but the concept is logical and these observations should be confirmed by other investigators. At the current time, the measurement of mRNA is labor-intensive and requires sophisticated laboratory experience. Because of the important relation between HPV and cervical carcinogenesis, a useful chemopreventive agent must suppress HPV expression. Therefore, HPV viral load and oncoprotein expression need to be developed extensively as biomarkers.

Oncogenes (ras, myc, c-erb B-2)

Ha-ras has been demonstrated to be overexpressed in cervical carcinoma and high-grade lesions compared with the normal cervix.[54–60] Several laboratories have identified H-ras codon 12 mutations in cervical carcinoma, but Van Le et al. were unable to find them in CIN lesions, suggesting that ras mutations are late events.[59]

c-myc was measured quantitatively and was shown to increase as lesions progressed from CIN to microinvasion.[33,61–63] Iwasaka et al. and Riou et al. demonstrated that c-myc overexpression is associated with disease recurrence and metastatic potential in invasive cervical carcinoma.[62,64]

c-erb B-2 was found in 39% of invasive cervical carcinoma cases by Hale et al., who noted a correlation with survival.[65] Mitra et al. evaluated a panel of 22 protooncogenes in 50 cervical carcinoma cases and found amplification of the genes for c-erb B-2 in 14%; amplification ranged from 5–68 copies, suggesting an important role in carcinogenesis.[66] c-erb B-2 oncoproteins were found to be overexpressed in cervical carcinomas, with pronounced overexpression in aneuploid tumors.[54,67,68]

All three oncogenes appear to be related to late events in cervical carcinogenesis. Their expression most likely is related to the genetic instability induced by HPV integration. These oncogenes most likely are not sufficiently expressed differentially to be of interest as biomarkers in chemoprevention trials.

Altered Growth Factors and Receptors

Several types of selected protein kinases and their receptors have been identified as being important in the development of cancer. The tyrosine kinase subfamily includes epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), platelet-derived growth factor, Src, Lck, and others. The serine kinase subfamily includes the transforming growth factor (TGF)-β receptor family. The cyclin-dependent kinase (Cdk) subfamily includes Cdk2, Cdc2, and others. Retinoic acid receptors (RARs) are intracellular receptors in the same class as cortisol, estrogen, progesterone, vitamin D, and thyroid hormone receptors.

Epidermal Growth Factor Receptor

erb B1 initially was discovered as one to two oncogenes carried by the avian erythroblastosis virus, for which the protooncogene was found to encode a membrane-associated tyrosine kinase protein that was identified later as the EGF receptor.[69–72] There is good consensus among immunohistochemical studies in the cervix that EGFR levels demonstrate a statistically significant increase as the severity of the lesion progresses from early dysplasia to invasion.[73–76] Two studies using homogenated cervical tissue found decreased expression of EGFR in tumors, but homogenated tissue may not be the best model for assessing the behavior of receptors in tissue.[77,78]

Several lines of evidence support the interaction of HPV with EGFR. When expressed from a heterologous promoter, the HPV E5 gene has been found to be oncogenic and to cooperate with EGF to enhance cellular transformation further.[79,80] Additional evidence from HPV-immortalized cell lines demonstrates that EGF suppresses insulin-like growth factor (IGF) binding protein 3, allowing the growth-potentiating effects of IGF-1 to predominate.[81]

In a previous chemoprevention trial, EGFR was not demonstrated to be modulated in the cervix.[82] However, Boiko et al. reported that pretreatment EGFR expression did predict histologic response in patients treated with DFMO.[82]

EGFR shows promise as a biomarker. Although EGFR was not modulated by DFMO, it may be modulated by other chemopreventive agents. It is expressed differentially in normal and high-risk tissue and appears at a well defined stage of carcinogenesis. EGFR is easily measured immunocytochemically and can be measured quantitatively using image analysis. However, to our knowledge large studies of its sensitivity, specificity, and accuracy have not yet been conducted in the cervix.

Vascular Endothelial Growth Factor

Vascular atypia is the hallmark of colposcopic progression of SIL to carcinoma. Vascular growth factors play an important biologic role. Fujimoto et al.[83] studied VEGF in the normal cervix and all cell types of invasive cervical carcinoma. They observed increases in VEGF, which correlated with microvessel counts in the tumors. In all cases, VEGF expression was less in the normal cervix than in the invasive tumors, but overlap in measurements was observed. In the current study, VEGF was measured using mRNA, a difficult technique. Because vascular proliferation is so clearly visible to the eye in the precursor SIL, VEGF may become an interesting biomarker. In our opinion, there currently are insufficient data concerning precursors, ease of measurement, and modulation in chemoprevention trials to recommend VEGF as a biomarker.

Transforming Growth Factor-β

The transforming growth factors-β constitute a family of local mediators that regulate the proliferation and functions of the majority of vertebrate cell types. The five members of the family, named TGF-β1 to β5, are proteins with similar structures and functions, but their effects on cells are quite varied. The receptors for members of this superfamily have been cloned and sequenced, and they are serine/threonine kinases.[84–86]

TGF-β1 inhibits proliferation of keratinocytes in normal epithelium. Loss of the peptide would be expected with increasing grades of CIN. Comerci et al. reported decreased expression of TGF-β1 in the epithelial component of CIN and invasive carcinoma, suggesting that the loss of the peptide may be an important event in early carcinogenesis.[87] Similar studies by Glick et al. in mouse papillomas suggested that decreased TGF-β 1 levels led to hyperproliferation and malignant progression.[88]

Cell line experiments have generated evidence that TGF-β 1, usually a growth inhibitor, in fact supports the growth of HPV type 16-immortalized cell lines by increasing EGFR under conditions supportive of squamous differentiation. TGF-β1 stimulates transcription of EGFR in several cell types. Woodworth et al. have demonstrated increased EGFR expression in immortalized, but not normal, cervical cells and verified their increased differentiation with involucrin and keratin expression.[84,86] Reconciling these findings in cell lines with those in tissue is difficult.

Comerci et al. studied TGF-β1 in a chemoprevention trial using β-carotene.[89] They reported significantly higher intracellular TGF-β1 immunoreactivity across parabasal, midepithelial, and superficial cell layers in cervical epithelial cells from 10 patients receiving β-carotene compared with cells from 10 control patients.[89]

Cyclin-Dependent Kinases and Cyclins

The cell cycle control system is based on two key families of proteins: Cdks and the cyclins. Cdks induce downstream processes by phosphorylating selected proteins on serine and threonine. Cyclins bind to Cdk molecules and control their ability to phosphorylate target proteins. There are two classes of cyclins: mitotic cyclins, which are required for entry into mitosis, and G_1 cyclins, which are required for entry into S-phase.[9,10]

E6 and E7 oncoproteins have been shown to functionally interfere with the Cdks $p16^{INK4}$, $p21^{C1P1}$, and $p27^{K1P1}$, apparently blocking their negative effect on regulation of the cell cycle. E6 has been shown to modify the activity of cyclins E and A, activating the expression of these cellular proteins. Thus, there is interest in the Cdks and cyclins with regard to biologic interaction with HPV.[9,10]

Kanai et al. demonstrated up-regulation in Cdks and cyclins in a series of 30 normal cervical specimens, 21 CIN specimens, and 33 invasive squamous cell carcinoma specimens.[90] No HPV typing was performed as part of the assessment, although we believe this would have been of interest. Although cyclins are of interest because of their interaction with E6 and E7, to our knowledge there are insufficient data to recommend their use as biomarkers at the current time.[90]

Retinoic Acid Receptors

RARs have been found to be important in predicting response to retinoids in tumors of the aerodigestive tract and have been shown to mediate decreased growth in cervical carcinoma cell lines treated with retinoids.[91] Cellular retinoid- and retinol-binding proteins (CRABP and CRBP, respectively) have been studied in CIN and invasive carcinoma. Hillemans et al. studied CRABP and CRBP and found that CRABP 1 was present in the basal layer of the normal cervix and low-grade CIN, but extended to the superficial layer in high-grade CIN and carcinoma.[92] Retinoid status has been found to control the appearance of reserve cells and keratin expression in mouse cervical epithelium.[93] Agarwal et al. proposed that retinoids act by reducing the extent of HPV viral oncogene transcription and thus slow the neoplastic process.[94]

Oridate et al. studied the effects of all-*trans*-retinoic acid, 12-*cis*-retinoic acid, and 4-hydroxyphenylretinamide (4-HPR) in eight cervical carcinoma cell lines.[95] Cell growth decreased in all lines, using all three medications. At some concentrations, apoptosis

was induced. Oridate et al. later demonstrated that 4-HPR induced apoptosis through a mechanism that was not dependent on RAR.[96] In the same study, treatment with oxygen-radical scavengers reduced reactive oxygen species and inhibited the 4-HPR-induced apoptosis, suggesting that a critical level of oxygen was needed to induce apoptosis.

Xu et al. were able to show differential expression of RAR and retinoid X receptor in premalignant fixed specimens of the cervix, comparing high-grade and low-grade areas. The expression of receptors decreased as lesions progressed.[97] If RARs are modulated in the cervix as they are in the aerodigestive tract, they may be reasonable biomarkers. However, further work needs to be done to verify their role in cervical chemoprevention trials.

Polyamines

Polyamines (putrescine, arginine, ornithine, spermidine, and spermine) play critical roles in cellular maintenance, proliferation, differentiation, and transformation. Ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, is considered to be a protooncogene that is crucial for the regulation of cellular growth and transformation.[98] Cancer patients have elevated levels of polyamines in their physiologic fluids compared with their healthy counterparts. DFMO, a specific "suicide inhibitor" of ODC, exhibits antitumor and antimetastasis activities and is effective in many carcinogen-induced animal chemoprevention models.[98] Preliminary studies by Nishioka et al.[99] indicated that ODC activity and polyamine levels could be measured with the amounts of tissue obtained by routine cervical biopsy (1.3–11.1 mg). However, they then observed the presence of cadaverine, which indicated bacterial contamination; this finding is not unexpected in the cervix, but it is a problem for polyamine measurement because some of the polyamines present might have come from the bacteria.[99] Thus, tissues were rinsed before freezer storage. As expected, increases in the plasma precursor amino acids of polyamines, such as arginine and ornithine, were observed, even at very low doses of DFMO. DFMO itself was measured as a compliance marker. The tissue measurements have shown wide variability, as expected and as noted in other organ sites in which DFMO studies have been undertaken.[100] Measurement of the tissue spermidine/spermine ratio has been evaluated as a way of decreasing variability and appears to be the most reliable of measurements.

Mitchell et al. showed that the tissue spermidine/spermine ratio and the plasma arginine level were modulated in a Phase I trial of DFMO treatment in patients with CIN. Polyamine biomarker response was dose related, but sample sizes were too small to examine reliably the relation between polyamine biomarkers and the histologic response.[101] Mitchell et al. calculated prospective sample sizes for chemoprevention trials given the variability of measurements in the cervix; recommended group sizes ranged from 12 (if using the spermidine/spermine ratio) to 80 (if using modulation of arginine).[102]

The relation between polyamines and HPV is unknown and needs further study because DFMO has been demonstrated to be a promising chemopreventive agent in the cervix.[101] Patients in the Phase I study responded regardless of HPV status, but only agents that decrease HPV expression will be of long-term interest as chemopreventives.

Polyamine markers are expressed in normal and high-risk tissue.[103] They have been demonstrated to be modulated in the cervix, colon, and skin by chemopreventive agents. However, they do not appear at a well defined stage of carcinogenesis. Because of their large variability, large sample sizes of specimens are required for their use in chemoprevention trials. In addition, highly specialized laboratories are required to obtain consistent results even when specimens are batched. Not until these markers can be more easily measured, with less variability, can they be recommended as biomarkers.

Arachidonic Acid

To our knowledge no studies have been published to date regarding the differential expression of arachidonic acid or prostaglandins in the cervix. Nonsteroidal antiinflammatory drugs (NSAIDs), which modulate arachidonic acid, have been found to be successful chemopreventive agents in organ sites such as the colon.[11,12] Studies of differential expression to validate these biomarkers will need to occur before trials of NSAIDs can take place in the cervix.

DIFFERENTIATION MARKERS

Differentiation markers include fibrillar proteins (keratins, involucrin, cornifin, filaggrin, actin microfilaments, microtubules), adhesion molecules (cell-cell: lectins, gap junction, desmosomes; cell-substrate: integrins, cadherins, laminins, fibronectin, proteoglycans, collagen), and glycoconjugates (mucins, blood group substances, and glycolipids).

Fibrillar Proteins

Keratins are proteins of 40–67 kilodaltons (kD) organized into filaments and found in different combinations in human epithelial tissues. Their expression correlates with distinct types of epithelial differentiation. Involucrin and cornifin are major protein con-

stituents of human cornified epithelium and undergo cross-linking by epidermal transglutaminase. Antibodies are available for studying keratins and involucrin in fixed tissue and transglutaminase in fresh tissue.

Several investigators have examined keratin expression in cervical epithelium.[104–111] The cervix appears to have lower molecular weight keratins (39 kD, 43 kD, and 58 kD) than, for example, the aerodigestive tract epithelium. Twenty keratins are modulated in the process of squamous differentiation.[112] Although these keratins can be studied immunohistocytochemically, their expression changes with cell layers (from parabasal to superficial) and as lesions progress in diagnosis (from normal cervix to SIL to carcinoma). To our knowledge the cytokeratin expression of the normal, dysplastic, and cancerous cervix has been the subject of two reviews.[112,113] The CAM 5.2 antibody binds to these keratins and is expressed differentially in normal, premalignant, and malignant epithelium. To our knowledge neither review addressed the relation between HPV and the differentiation process. The relation between HPV infection and cytokeratin differentiation is complicated and poorly understood.[112,114]

Conversely, involucrin, as measured by immunoperoxidase staining by two groups of investigators, is present in nearly all normal epithelium and HPV lesions but disappears as the lesions progress to CIN and invasive carcinoma.[115–117] Statistically significant decreases in involucrin were noted as the cervical lesions progressed to invasion in a preliminary study of 23 cone biopsy specimens that ranged from CIN-1 to carcinoma.[97] Statistically significant decreases in cornifin also were noted.[97]

Keratins, involucrin, and cornifin are of interest as markers because they are modulated by retinoids.[118–120] Differential expression of keratins and involucrin has been demonstrated in cervical carcinoma cell lines.[120] Filaggrin, a differentiation-dependent cytoplasmic protein, is altered in cells with HPV, and its expression decreases as lesions progress to invasive carcinoma.[55]

Although these markers are expressed differentially in normal and high-risk tissue, to our knowledge they have not yet been shown to appear at a well defined stage of carcinogenesis. Although they can be measured immunohistocytochemically, the variation reported in the literature would suggest that their measurement is not easy. To our knowledge to date large studies documenting their sensitivity, specificity, and accuracy have not been performed. In addition, their relation with HPV is unknown, and they have not been shown to be modulated by chemopreventive agents. They most likely will not have as much potential as biomarkers if the technical issues with HPV measurement cannot be resolved.

Adhesion Molecules

Discrete combinations of adhesion molecules are expressed by endothelial cells at different anatomic sites, and these combinations appear to be responsible for the selective recruitment of different leukocyte subpopulations into particular tissues. The up-regulation of adhesion molecules by locally released soluble mediators is an important process in enabling the focusing of leukocytes in large numbers at sites of inflammatory or immunologic activity. Adhesion molecules are able to interact specifically with ligands expressed on the surface of vascular endothelial cells, which include members of the selectin family, such as E-selectin and P-selectin, and those belonging to the immunoglobulin superfamily, principally ICAM-1, ICAM-2, and VCAM-1. Vascular adhesion molecules are up-regulated in high-grade CIN lesions but not in low-grade lesions.[121] Epithelial adhesion molecules show increased expression as cervical lesions progress.[122]

Integrins are transmembrane glycoprotein heterodimers comprised of noncovalently associated α and β subunits, which link the cytoskeleton to the extracellular matrix. The extracellular domains of both subunits contribute to the ligand-binding site whereas the cytoplasmic domains interact either directly or via linker molecules with the actin cytoskeleton. Three major integrins have been described in epithelial cells: $\alpha 2\ \beta 1$, $\alpha 3\ \beta 1$, and $\alpha 6\ \beta 4$. In squamous epithelium, β integrins are involved in cell-cell contacts, whereas $\alpha 6\ \beta 4$, a component of hemidesmosomes that may function as a laminin receptor, is involved in the formation of bonds between epithelial cells and basement membranes. Derangement in the expression of the tissue-specific integrin complement may play a key role in epithelial neoplastic progression because cell-cell contact affects both differentiation and invasive ability. Integrins $\beta 1$ and $\beta 4$ have enhanced expression in high-grade lesions and tumors.[123–125] The differential expression of laminins and fibronectin has been demonstrated in cervical carcinoma cell lines but to our knowledge has not yet been demonstrated as a useful marker in tissues with CIN.[120]

Although these markers are expressed differentially in normal and high-risk tissue, to our knowledge they have not yet been shown to appear at a well defined stage of carcinogenesis. They can be measured immunohistocytochemically, but not easily. To our knowledge large studies documenting their sensitivity, specificity, and accuracy have not been performed. In addition, to our knowledge their relation

with HPV is unknown, and they have not been shown to be modulated by chemopreventive agents. They most likely will not have as much potential as biomarkers if the technical issues with HPV measurement cannot be resolved.

Glycoconjugates

Lectins and glycoconjugates are aberrantly expressed as neoplastic transformation advances. In their studies of jack fruit lectin in exfoliated cervical cells of increasing grades of CIN, Pillai et al. found no binding in normal cells and intense binding in highly dysplastic cells.[126] Li studied lectin receptors in normal, dysplastic, and neoplastic cervices with a panel of 12 lectins; some receptors were found to correlate with tumorigenicity, others with differentiation, and others with invasion.[127] Lectins are of interest as markers because they are modulated by retinoids.[128–130] CD44 is a glycoprotein that functions as a surface receptor for the extracellular matrix glycan hyaluron, which mediates important aspects of lymphocyte activation and cellular migration. Woerner et al. and Kohlberger et al. demonstrated a statistically significant negative correlation between CD44 and increasing grade of CIN and carcinoma.[131,132]

Although these markers are expressed differentially in normal and high-risk tissue, to our knowledge they have not yet been shown to appear at a well-defined stage of carcinogenesis. Large studies documenting their sensitivity, specificity, and accuracy have not been performed. The relationship with HPV is unknown, but if they can be shown to correlate with the immunobiology of HPV, they may be of interest as biomarkers in vaccine trials.

GENERAL GENOMIC INSTABILITY MARKERS

General genomic instability may be the most important biological marker of all, because it may reflect the sum of the changes in all other categories. Aneuploidy is a well established marker of prognosis in other organ sites. Increasingly, in tumors with a field effect (e.g., those occurring in the aerodigestive tract), general genomic instability of the field has been demonstrated.

Chromosome Aberrations

The cell nucleus contains large loops of DNA whose ribosomal RNA genes are transcribed by RNA polymerase I; such a loop is known as a nucleolar organizer region (NOR). In humans, NORs are located on the secondary constrictions of acrocentric chromosomes. In the diploid cell, it is possible to see up to 10 NORs. NOR-associated proteins can be stained with silver (AgNORs). AgNORs increase as the lesions progress from koilocytosis to high-grade CIN. Authors differ with respect to their correlation with proliferation.[29,133,134]

Micronuclei are intracytoplasmatic inclusion bodies formed from chromatin fragments or whole chromosomes. Their presence in cells is a reflection of chromosomal aberrations during cellular mitosis. The micronuclei in exfoliated cells of the cervix have been suggested as a marker of malignant potential. CIN-2 and CIN-3 were shown to have higher levels of micronuclei than CIN-1.[135]

Three-group metaphases are morphologically well defined and readily recognizable (by light microscopy) atypical mitotic figures and are associated with aneuploidy. In a study of 72 cone specimens containing CIN, Pieters et al. demonstrated increased 3-group metaphases in women with higher grade CIN, in women with aneuploid lesions, and in women age > 35 years.[136]

Although these markers are expressed differentially in normal and high-risk tissue, to our knowledge they have not yet been shown to appear at a well-defined stage of carcinogenesis. Although they are measured immunohistocytochemically, the variation in reports in the literature would suggest that their measurement is not performed easily. Large studies documenting their sensitivity, specificity, and accuracy have not been performed to our knowledge. In addition, their relation with HPV is unknown, and to our knowledge they have not been shown to be modulated by chemopreventive agents. They most likely will not have as much potential as biomarkers if the technical issues with HPV measurement cannot be resolved.

DNA Abnormalities

DNA methylation is used in vertebrate cells mainly to insure that once a gene is turned off, it stays completely off. Global DNA hypomethylation has been observed in some human neoplasms and has been implicated as an important factor in carcinogenesis. Kim et al. examined whether DNA hypomethylation occurs in CIN and carcinoma and determined the relation between the degree of DNA methylation and the grade of neoplasia. Methylation was measured by ^{1}[H]methyl-group incorporation and was found to be increased threefold and sevenfold in the DNA from cervical dysplasias and carcinomas, respectively, compared with normal cervical tissues.[137] DNA hypomethylation was confirmed by the same group in a subsequent case series of specimens ranging from normal cervix to CIS. Hypomethylation was demonstrated to be significantly different between normal cervical and CIN-1 specimens and between normal

cervical and CIS specimens. Serum and tissue folate concentrations were found to be correlated positively with the degree of hypomethylation (correlation coefficient = 0.431; $P < 0.001$). Tissue folate concentrations increased steadily, whereas more variability was noted in serum levels.[138]

Loss of heterozygosity (LOH) studies reveal losses of genes at specific chromosomes that are commonly altered in human tumors.[138–140] These changes are believed to be critical for unmasking the recessive genetic changes of carcinogenesis. Yokota et al. demonstrated LOH at the D3S2 locus on chromosome 3p in nine fresh cervical tumors. This locus also commonly is lost in lung carcinoma and renal cell carcinoma.[139] Similarly, Chung et al. found LOH at 3p25 and 3p14.[140] Mitra et al. performed detailed allelotype analysis of DNA from 53 primary cervical tumors and corresponding normal cells using 57 polymorphic probes mapped to each of the chromosomal arms, excluding the short arms of the acrocentric chromosomes. They observed LOH at sites on 11 chromosomal arms: 1q (in 26% of tumors), 3p (35%), 4q (46%), 5p (53%), 5q (38%), 6p (28%), 10q (28%), 11p (42%), 18p (38%), and Xq (26%). The most frequent LOH in their study was found on 4q (ADH3) and 5p (D5S19).[141] LOH may not in itself be a biomarker but it may help researchers to understand the process of carcinogenesis, thus allowing the discovery of other biomarkers.

Heselmeyer et al.[142] reported a gain of chromosome 3q defining the transition from severe dysplasia to invasive carcinoma in a series (3 normal cervical specimens, 4 CIN-1 specimens, 6 CIN-2 specimens, 13 CIN-3/CIS specimens, and 10 invasive carcinoma specimens) in which comparative genomic hybridization was used to investigate chromosomal aberrations. The tissues were characterized further with regard to ploidy using DNA cytometry, HPV sequences using PCR, and proliferation using the MIB-1 antibody immunohistochemically. Chromosome 3q was overrepresented in 90% of the carcinomas and was found to be amplified. The gain of chromosome 3q was present in HPV type 16-infected aneuploid cells. This small study suggests a pivotal role for this genetic aberration in cervical carcinogenesis.[142]

Although these markers are expressed differentially in normal and high-risk tissue and have been shown to appear at a well defined stage of carcinogenesis, they are not easily measured. To our knowledge large studies documenting their sensitivity, specificity, and accuracy have not been performed. Their relation with HPV-induced carcinogenesis will be their major source of interest. To our knowledge to date they have not been demonstrated to be modulated by chemopreventive agents in the cervix.

Aneuploidy

Additional evidence for the histopathologic continuum of CIN to invasive carcinoma has been found in DNA ploidy analyses using flow cytometry.[143–153] In flow cytometry, a DNA histogram is generated for each sample. Many studies using flow cytometry showed aneuploidy to be an important predictive factor for the progression of cervical lesions. These studies preceded the use of computer-assisted image analysis.

Despite the consistent finding in other tissues that aneuploid lesions had a worse prognosis, there remain broad differences in estimates of aneuploidy among studies of cervical lesions. In some of these studies, flow cytometric measurement was compared with computer-assisted image analysis. Three studies examining ploidy with flow cytometry in CIN-3 lesions reported wide ranges (range, 20–80%) of aneuploidy.[144–148] Hughes et al.[146] reported that aneuploidy was no more common in CIN-3 than in HPV-infected cervices (21% vs. 18%). Willen et al.[147] reported that only 50% of invasive lesions were aneuploid. Watts et al.[148] compared flow cytometry with image analysis in specimens divided into two groups and demonstrated that image analysis had greater sensitivity than flow cytometry. They reported that many of the HPV lesions without CIN were aneuploid (47% with flow cytometry, 87% with image analysis). This may be considered as evidence that HPV infection truly is part of the pathologic continuum to carcinoma. Hanselaar et al. demonstrated DNA aneuploidy in 89% of cervical samples from women with CIN-3 with or without adjacent invasive carcinoma. The DNA patterns in the areas of CIN and invasive carcinoma were identical, suggesting that the lesions were related.[145] HPV appears to alter the DNA index sufficiently so that HPV always should be measured.

Park et al. showed that low-risk HPV types tend to be associated with polyclonal lesions whereas high-risk HPV types are associated with monoclonal lesions.[153] Boiko et al. reported findings suggestive of the elimination of aneuploid clones using quantitative image analysis in the Phase I DFMO trial.[21] Ploidy appears to be a measurable and modulable biomarker.

Ploidy appears to be a good predictor of biologic behavior and may have better predictive value than histopathologic characteristics as judged macroscopically. The aforementioned studies of ploidy did not measure HPV consistently, which appears to affect ploidy. These studies need to be validated with larger samples, placing emphasis on consistent histopathologic review, controlling for HPV as measured by

quantitative PCR, and using image analysis rather than flow cytometry because it is quantitative and reproducible. Genomic instability is a risk factor for invasive carcinoma because these abnormalities drive the multistep carcinogenesis process.

TISSUE MAINTENANCE MARKERS

The explosion in knowledge of the biology of cancer leaves us with some markers that are applicable to more than one category. These markers have been designated tissue maintenance markers for the current review.

Metalloproteinases

The maintenance of tissue organization, including cellular proliferation and migration, is mediated in part by the extracellular matrix. The extracellular matrix juggles formation and degradation and is regulated under normal conditions by the matrix metalloproteinases (MMPs), a zinc-dependent and calcium-dependent enzyme family. The MMPs include 14 members, grouped by substrate (gelatinases, interstitial collagenases, stromelysins, elastases, and membrane-type MMPs). These enzymes have been studied intensively and have been found to correlate with invasive potential in breast carcinoma and hepatocellular carcinoma.[154,155] Yang et al. found that the nuclear matrix proteins p69, p186, and p200 have increased affinity for binding HPV type 16 in cervical carcinoma cell lines compared with other nuclear matrix proteins.[156] Five studies showed progressive increases in staining for MMPs as the tissues progressed from normal to SIL to cervical carcinoma.[157–161] Only one study[157] examined HPV positivity of the tissue, and the number of specimens was small (18 HPV positive tissues and 11 HPV negative tissues). A nonsignificant difference in staining was noted. MMP staining in all studies progressively increased as the grade of the lesion or stage of the tumor increased.

Although these markers are expressed differentially in normal and high-risk tissue, to our knowledge they have not yet been shown to appear at a well-defined stage of carcinogenesis. Similar to polyamine biomarkers, they can be studied in plasma and tissue, which increases their potential usefulness in clinical trials. Large studies documenting their sensitivity, specificity, and accuracy have not been performed to our knowledge. In addition, their relation with HPV is unknown, but if related, they would be of greater interest. They most likely will not be found to have as much potential as biomarkers in chemoprevention trials but may serve as biomarkers of invasive potential in chemotherapy trials.

Telomerases

Telomerases are ribonucleoprotein enzymes capable of extending chromosome ends with specific telomeric sequences. These enzymes allow cells to proliferate indefinitely. Telomerase activity usually is present in the germline and in the majority of human tumor tissues. Telomerases help control the number of genetic defects, which, as they accumulate over time, combine to bring about disruption of growth and malignancy.[162–164] Kawai et al. reported high telomerase positivity in cervical carcinoma specimens, cervical swabs from patients with HGSIL, and patients who were HPV positive.[163] Shroyer et al. showed positivity in 18% of 50 normal cervices, 56% of 18 reactive atypias, 56% of 25 LGSILs, 96% of 26 HGSILs, and 100% of 18 invasive tumors. The specimens were not HPV typed.[164] Nagai et al. reported similar results in 100 cervical biopsies (19% of 16 normal cervices, 31% of 25 CIN-1 specimens, 50% of 6 CIN-2 specimens, 60% of 30 CIN-3 specimens, and 92% of 23 invasive carcinomas), which also were not HPV typed).[162]

Although these markers appear to be expressed differentially in normal and high-risk tissue, they also are positive in HPV-positive patients. These few small studies may be confounded by HPV, which appears to induce telomerase activity. To our knowledge telomerase activity has not been studied in chemoprevention trials, nor have a sufficient number of studies of its quantitation been performed to allow its use as a biomarker. Telomerase activity may be helpful in elucidating the mechanisms by which HPV immortalizes cells.

Apoptosis and Antiapoptotic Markers

Apoptosis, or programmed cell death, causes the cell nucleus to shrink and condense. By contrast, cell death by cellular necrosis leads cells to swell and burst. The apoptotic cell disappears rapidly, phagocytosed by macrophages.[165–167]

BCL-2 is an inhibitor of apoptosis. Ter Harmsel et al. studied the presence of BCL-2 in SILs and cervical carcinomas and found increased staining for BCL-2 as the lesions progressed.[165] This finding suggests that as cervical lesions progress they become increasingly resistant to apoptosis. This hypothesis is supported by experiments in cervical carcinoma cell lines. Kim et al. reported that p53 and apoptosis are induced by hypoxic conditions in cervical carcinoma cell lines. E6-immortalized and E7-immortalized cell lines have demonstrated decreased apoptosis, indicating that these immortalized cell lines have acquired genetic alterations that decrease sensitivity to apoptosis.[166] Yang et al. demonstrated a similar resistance to apoptosis when E6-immortalized and E7-immortalized cell lines were exposed to cigarette condensate carcinogens.[167]

TABLE 2
Summary of Surrogate Endpoint Biomarkers in the Cervical Epithelium

SEB	Relation to HPV studied	Differentially expressed in normal and premalignant tissue	Appears at a well defined stage of carcinogenesis	Well studied sensitivity, specificity, and accuracy	Easy to measure	Modulated by chemopreventive agents	Correlated with decreased cancer survival or increased cancer incidence
Quantitative cytology and histopathology		+	+	+	+	+	+
PCNA		+	+		+	+	
Ki-67, MIB-1	+	+					
Labeling indices		+	+		−		
MPM-2		+	+		+	+	
p53, Rb		+					
HPV viral load	+	+	+			+	
Oncogenes	+	+					
EGFR		+	+		+	−	
VEGF		+					
TGF-β	+	+					
Cdks		+					
RAR		+	+				
Keratins		+					
Involucrin		+	+				
Cornifin		+	+				
Adhesion molecules		+					
Glycoconjugates		+					
AgNORs		+					
Micronuclei		+					
Three-group metaphases		+					
DNA hypomethylation		+					
LOH		+					
Gain in chromosomes		+					
Aneuploidy by flow cytometry	+	+	+		+		+
Metalloproteinases		+					
Telomerases		+					
Apoptotic markers		+					

SEB: surrogate endpoint biomarkers; HPV: human papillomavirus; +: studies showed a positive relation; PCNA: proliferating cell nuclear antigen; −: studies showed no relation; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; TGF-β: transforming growth factor-β; Cdks: cyclin-dependent kinases; RAR: retinoic acid receptors; AgNORs: silver-stained nucleolar organizer region proteins; LOH: loss of heterozygosity.
Empty cells indicate that no information was available.

Although these markers are expressed differentially in normal and high-risk tissue, to our knowledge they have not yet been shown to appear at a well-defined stage of carcinogenesis. Experimental evidence from cell lines suggests there may an interesting relation between apoptosis and antiapoptotic markers and HPV. To our knowledge no studies have been performed to date using these markers in chemoprevention trials, but they would be of great biologic interest. Chemopreventive agents that could induce apoptosis would be very useful.

CONCLUSIONS

Many SEBs have been studied in cervical tissue. Their potential suitability for use in chemoprevention trials in the cervix is summarized in Table 2. For a few of these SEBs, a rationale has been derived from studies of cervical carcinoma cell lines. However, in few has their relation with HPV been explored or their relation with the carcinogenic aspects of HPV established. The majority of those markers reviewed, with the exception of several oncogenes, were expressed differentially in normal and premalignant cervical tissue. The oncogenes appeared in cancerous lesions only and thus are involved late in the carcinogenic process. To our knowledge only aneuploidy has been well established as a marker of cancer incidence and mortality.

Those biomarkers that appear to be most promising are those that can be measured quantitatively (quantitative cytologic and histopathologic nuclear

measurements, PCNA, MIB-1, MPM-2, HPV viral load, EGFR, polyamines, and ploidy). Many of these same promising markers have been demonstrated to be modulated in chemoprevention trials in the cervix (histopathologic nuclear measurements, PCNA, MPM-2, polyamines, and HPV viral load). The markers of the most interest in the future will be those that are shown to 1) correlate well with the biology of HPV, 2) be modulated by several chemopreventive and immunopreventive agents, 3) have limited variability in repeated measurement, allowing small sample sizes, and 4) be suitable for automated procedures that lead to ease in measurement.

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