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Intraepithelial Neoplasia, Surrogate Endpoint Biomarkers, and Cancer Chemoprevention

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Abstract Neoplasia is a progression of molecular, cellular, and tissue changes starting with a critical cell mutation and advancing by clonal evolution, involving further multiple mutations and expanding mutated clones. This process is characterized by five general stages: latency, focal growth of normal-appearing but disorganized cells, abnormal-appearing cells (dysplasia), microinvasion, and finally, metastasis. The two driving forces of neoplastic progression in an epithelium are mutagenesis and mitogenesis. These forces frequently occur concurrently, produced by exposure of the epithelium to environmental and endogenous mutagens and mitogens. The major strategy of chemoprevention is to block the effects of both mutagens and mitogens during the early stages of predysplasia and dysplasia.

Surrogate endpoint biomarkers (SEBs) are tissue, cellular, and molecular changes that correlate with the later development of cancer. Because of the savings in cost, labor, and time, SEBs are urgently needed to replace the use of cancer incidence reduction as the endpoint for chemopreventive agent clinical trials. The advent of computer-assisted cytometry allows each of the seven basic criteria of dysplasia to be individually assayed as an SEB. Since the dysplastic changes that characterize intraepithelial neoplasia are embodied in the causal pathway to invasive neoplasia, they are already validated as predictors of cancer incidence. More attention should be paid to the quality control of SEB assays, including control of variation in cell composition of tissue samples, assay protocol, instrumentation used, and observer performance. The dose-response relationship between a known chemopreventive agent and the SEB should also be evaluated.

The Division of Cancer Prevention and Control, National Cancer Institute, has begun a program to test chemopreventive agents in short-term Phase II clinical trials using dysplasia-based SEBs. The SEBs are assayed, when possible, by computerized cytometry. Trials are being conducted for oral leukoplakia, cutaneous actinic keratosis, superficial bladder cancer, pulmonary metaplasia/dysplasia, cervical dysplasia (CIN III), and adenomatous colonic polyps.
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DEFINITION OF NEOPLASIA

Neoplasia is defined as an abnormal tissue condition characterized by the gradual progression of cell mutations and unregulated clonal

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expansion to form an expanding, disorganized mass. This mass distorts adjacent normal tissue by compression and/or infiltration, and disseminates proliferating neoplastic cells to other parts of the body.

STAGES OF NEOPLASIA

It is generally accepted that neoplasia begins with a critical mutation in a cell which initiates continuous and unregulated clonal proliferation. The cells form an abnormal disorganized structure which characteristically distorts adjacent normal tissue. Initially, the cells are normal in appearance, but in time, further mutations (occurring either spontaneously secondary to increased genetic instability or as the result of continuing exposure to carcinogens) cause clearly abnormal morphological changes in cells as well as tissue patterns. The abnormal cytomorphologic and morphologic criteria of dysplasia may be reduced to seven basic changes: increased nuclear size, altered nuclear shape, increased nuclear stain uptake, nuclear pleomorphism (increased variation in size, shape, and stain uptake), increased mitoses, abnormal mitoses, and disordered or absent differentiation to mature epithelial structure. The neoplastic cell clones continue to proliferate and expand within the epithelium, and eventually push or infiltrate across the basement membrane. At the point of microinvasion, the diagnostic nomenclature used by pathologists changes dramatically. Prior to invasion the process is called *intraepithelial neoplasia* and is characterized as benign, whereas after invasion the same process is called *cancer* or *carcinoma* (epithelial cancer) and is characterized as malignant. After invasion, the descriptive term *dysplasia* is little used and tends to be replaced by the term *anaplasia*.

Neoplasia is thus a continuous gradual progression of molecular, cellular, and tissue changes which advance with increasing deviation from normal structure and function. This progression may be divided into five general stages, based on the microscopic appearance of the neoplastic population and its relationship to the basement membrane.

The Latency Stage

This is the period between the first critical cell mutation and a microscopically visible focus of clonally expanding mutated cells. During this stage the epithelium appears microscopically normal, but at the molecular level critical mutational and functional events have occurred and may still be occurring.

The Stage of Focal Growth of Normal-Appearing but Disorganized Cells

This stage starts with the first microscopic appearance of an expanding intraepithelial focus of neoplastic cells. The neoplastic cells are individually normal in appearance but are arranged

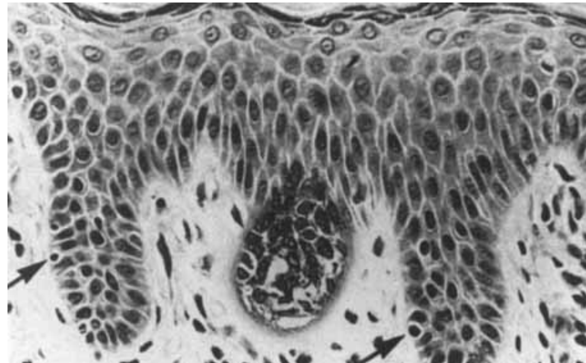

Fig. 1. Epidermis showing a focus of dysplastic melanocytes representing the dysplastic stage of intraepithelial neoplasia. Normal melanocytes are indicated by arrows. From a patient with Dysplastic Nevus Syndrome.

in a disorganized tissue pattern. The existence of disorganized but normal-appearing cells is characteristic of many benign neoplasms, particularly of glandular tissue (adenomas), as well as of fibromas, myomas, and lipomas. It should be noted that although the cells of this stage are normal in appearance, their clonal pattern, abnormal organization, and tendency to distort adjacent normal cells mark them as neoplastic. An example of this stage is the "aberrant crypt" which appears in the colon of rats soon after they have been fed dimethylhydrazine [2]. The individual cells in aberrant crypts are normal in appearance but form a disorganized and distorted crypt-like structure which tends to compress adjacent normal cells. Later, dysplastic cells appear, and the aberrant crypts are at high risk for developing into invasive adenocarcinoma [3].

The term "predysplastic stage of neoplasia" is commonly used to describe the combined stages of latency and of clonal expansion of normal appearing but disorganized cells.

The Stage of Dysplasia

This stage begins with the first appearance of characteristic abnormal cytomorphological changes, called "dysplasia" (see below), and extends to the point of invasion across the basement membrane. Figure 1 illustrates an expanding nest of dysplastic neoplastic melanocytes during this stage of intraepithelial neoplasia of the skin. The nest is one of many similar skin lesions of a patient with Dysplastic Nevus Syndrome. This intraepithelial neoplasm is at high risk for developing into invasive cancer, shown by the increased incidence of melanoma (3.5%) in patients with Dysplastic Nevus Syndrome [6]. The monoclonal origin of the cells in the nest may be inferred not only from their arrangement but also from the extensive published evidence for the monoclonal origin of the majority of neoplasms [7]. Figure 2 illustrates growth patterns that may be exhibited by intraepithelial neoplasms. They may expand laterally with compressing or pushing borders (Fig. 2a) or with infiltrating borders (Fig. 2b). They may extend upward to involve the full thickness of the epithelium (Fig. 2c) or downward across the basement membrane (Fig. 2d) [7].

The preinvasive stage of neoplasia is synonymous with the stage of intraepithelial neoplasia and is made up of the latency, clonal expansion of normal-appearing but disorganized cells, and dysplastic stages.

The Stage of Invasion

The invasion stage begins when the expanding neoplastic cell population pushes or infiltrates across the basement membrane. The lesion is now called cancer, malignant, or carcinoma (epithelial cancer). Customarily, from the point of microinvasion onwards, the term "dysplasia" is no longer used to describe the neoplastic process.

The Metastatic Stage

The metastatic stage is defined as the period after invasion and starts with the appearance of neoplastic cells disseminating away from the primary tumor mass singly and/or in groups, either by permeation through tissue spaces or via transport in lymphatics and blood vessels, to set up secondary growths (metastases) in other parts of the body.

GRADING AND STAGING

In general, similar to the grading and staging of a macroscopic neoplasm seen clinically, an intraepithelial neoplasm may be assigned a *grade* based on the degree of its cytological dysplasia, and a *stage* based on the extent of its spatial distribution. Conventionally, the stages of intraepithelial neoplasia are categorized by pathologists as mild—involving less than one-third of the thickness of the epithelium; moderate—involving between one-third and two-thirds thickness; and severe—involving between two-thirds and full thickness. Unfortunately, a system that explicitly includes both the grade, based on cytomorphotologic aberration, and the microstage, based on extent of lesion, is not in general use.

Full thickness lesions are not infrequently called "carcinoma *in situ*." This name has been criticized because the condition it purportedly describes cannot be distinguished from severe dysplasia [8,9]. More importantly, the term "carcinoma *in situ*" infers that the neoplastic process actually starts at this point and that the preceding morphological changes of dysplasia

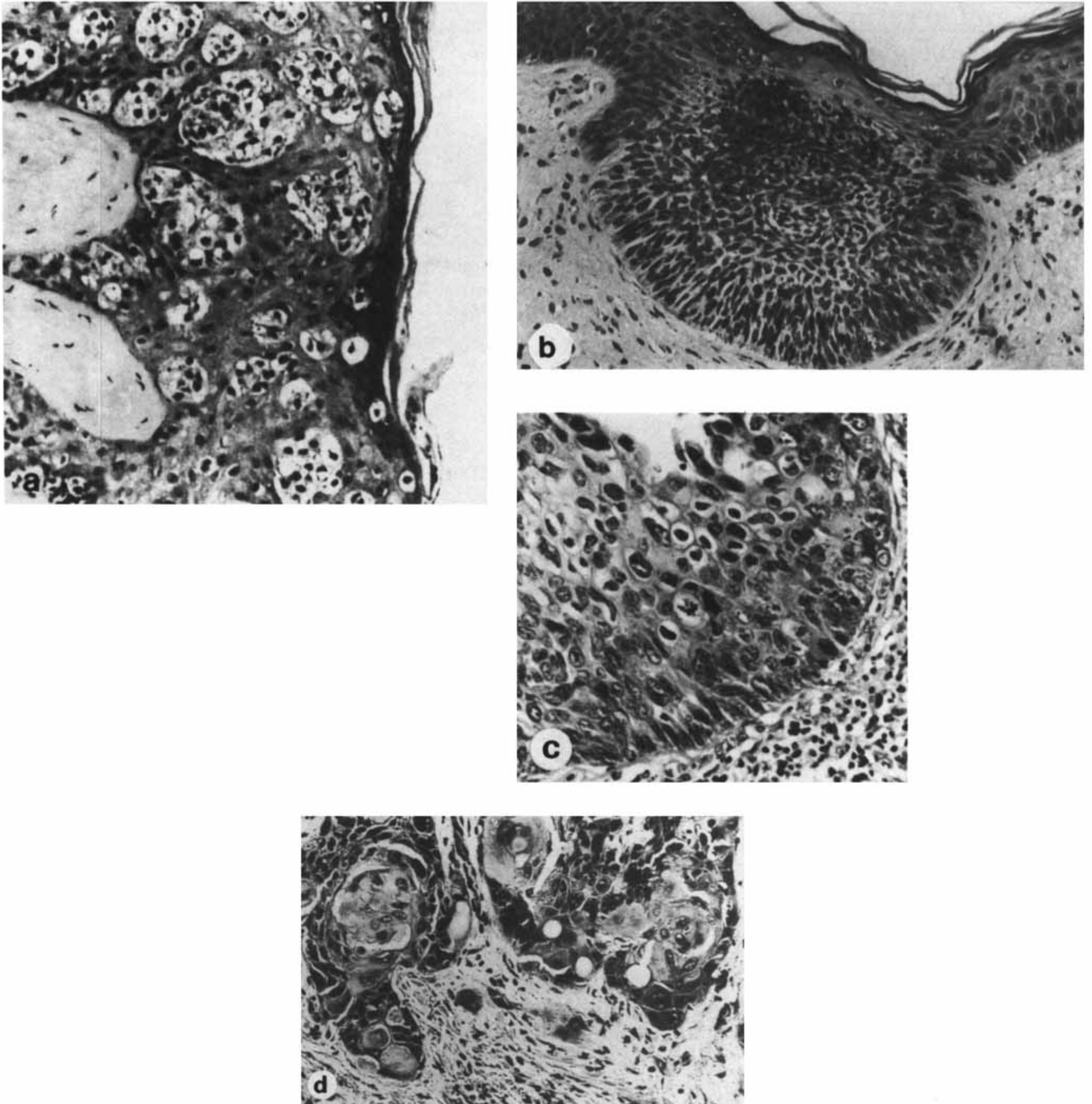

Fig. 2a. Basal cell epithelioma of the skin, an intraepithelial neoplasm composed of epidermal basal cells. The lesion exhibits a pushing border that compresses adjacent normal tissue cells

Fig. 2b. Superficial spreading melanoma, an intraepithelial neoplasm derived from melanocytes. The neoplastic cells are spreading by infiltration through adjacent normal epithelium. In this case, the risk of potential invasion is so high that the lesion is considered malignant.

are somehow "preneoplastic." On the contrary, the neoplastic process starts with the first critical mutation of a single cell. Foulds [10] and others [1] have emphasized that it is a serious error to call dysplasia (intraepithelial neoplasia) "preneoplastic" rather than neoplastic. This issue is important; calling dysplasia "preneoplastic" introduces the patently false and misleading idea that the entire process of intraepithelial neoplasia is not neoplasia, but some supposed precursor condition, and that neoplasia begins only when dysplastic cells are found invading across the basement membrane. The preferred alternative terms for dysplasia (aside from intraepithelial dysplasia) are "precancerous" or "preinvasive neoplasia."

The predysplastic and dysplastic stages of intraepithelial neoplasia are the primary targets for chemoprevention. Chemopreventive drugs are more likely to be effective during these early preinvasive stages than at the later postinvasive stages, when neoplasms become large enough to be clinically detectable. The earliest detectable lesion generally has an estimated volume of 1 ml and contains approximately 10^9 cells [5]. Such a macroscopic neoplasm is likely to contain a large number of mutant clones potentially capable of resisting the action of chemopreventive agents [5].

CLONAL EVOLUTION DURING INTRAEPITHELIAL NEOPLASIA

The phenomenon of clonal evolution in tumors was originally described by Nowell [11] as the continuous *variation* of cell phenotypes in the neoplastic population due to continuously occurring cell mutations associated with *selection* and clonal overgrowth of the fastest growing mutant phenotypes. A memorable descriptive phrase for this process is "neoplastic microdarwinism." The pace of cellular mutation and clonal selection may vary with the health of the epithelium involved and its exposure to environmental and endogenous mutagens and mitogens (see below).

THE DRIVING FORCES OF NEOPLASTIC PROGRESSION: MUTAGENESIS AND MITOGENESIS (WHICH FREQUENTLY OCCUR CONCURRENTLY DUE TO SIMULTANEOUS EXPOSURE TO MUTAGENS AND MITOGENS)

Epithelia undergoing neoplastic progression are frequently exposed to mutagens and mitogens simultaneously. In the common case of continuous exposure to polycyclic aromatic hydrocarbons derived from the combustion products of fossil fuels and tobacco, the same molecules frequently induce both mutagenesis and mitogenesis. Benzo(*a*)pyrene, for example, is an intense stimulant of hyperplasia [12]. The important relationship between concurrently occurring mutagenesis and mitogenesis on the one hand, and the rate of neoplastic progression through clonal evolution on the other (see Fig. 3) has been described previously [1]. This relationship suggests that chronic exposure of intraepithelial neoplasia to mutagens speeds the rate of neoplastic progression. This was experimentally established by the extensive work of Druckrey [13] who showed, for a number of carcinogens over a wide range of doses, cancer types, and animal species, that when animals were given a daily dose of carcinogen, the lengths of time to 50% cancer incidence became shorter as the dose of carcinogen was increased. Druckrey demonstrated a precise analytical relationship between the daily dose of carcinogen (d) and the time to 50% tumor incidence (t) as dt^n = a constant, where n is related to the number of critical mutations in the neoplastic population and its proliferation rate [13]. Druckrey's findings confirm what appears to be common sense, *i.e.*, the rate of clonal evolution in a neoplastic epithelium (which parallels the rate of neoplastic progression) speeds up when the epithelium is exposed to an increased chron-

Fig. 2c. Severe intraepithelial neoplasia of lung respiratory epithelium showing extension to full thickness of the epithelium.

Fig. 2d. Epidermis showing microinvasion of neoplastic cells across the basement membrane, justifying a diagnosis of squamous cell carcinoma.

ic dose of mutagen (carcinogen) because the cell mutation rate is increased.

The rate of neoplastic progression is also increased by chronic exposure of intraepithelial neoplasia to mitogens. This was established by Burns [15] who used the DMBA/TPA skin paint system in mice to show that the latent period before the appearance of carcinomas was shortened when the dose of TPA, a practically pure mitogen, was increased. The acceleration may occur (a) because the S-phase fraction is increased, and S-phase cells are at least ten times more vulnerable to mutation due to the absence of repair enzymes [16], and (b) because the rapid clonal expansions that occur expose larger targets of proliferating cells to mutagen fluxes.

The concept that concurrent and continuous mutagenesis and mitogenesis drive the rate of neoplastic progression (by stimulating the rate of clonal evolution) is important to the chemoprevention field because it clearly substantiates the idea that drugs which block mutagenesis, mitogenesis, or both will very likely have chemopreventive activity.

THE QUIET REVOLUTION IN DIAGNOSTIC HISTOPATHOLOGY: COMPUTER-ASSISTED IMAGE CYTOMETRY AND FLOW CYTOMETRY

While light microscopy will no doubt continue to be the mainstay of tissue diagnosis for some time to come, the advent of computer-assisted cytometric techniques is quietly revolutionizing the practice of diagnostic histopathology. The sensitive and precise measurements of the morpho- and photometric parameters of dysplasia made by the computerized image cytometer, when compared to the present-day alternative of having a microscopist subjectively estimate such relatively crude categories as "moderate to severe," "pleomorphism," or "hyperchromasia," offer a diagnostic capability that is greater by several orders of magnitude.

There are two techniques of cytometry based on how the tissue samples are prepared and assayed: image (or static) cytometry, and flow cytometry. Flow cytometry is faster and less labor-intensive, but requires that the tissue sample be disaggregated to obtain a homogenous suspension of cells. For purposes of SEB development, image cytometry is preferred because the tissue sample is intact and important information relative to tissue architecture and the degree of neoplastic progression is therefore retained. In addition, image cytometry may be performed on very small biopsy samples. For these reasons, image cytometry will be the principle topic considered here. However, except for the loss of intact tissue samples, the principles of computerized image cytometry discussed below apply equally as well to flow cytometry.

Basically, two modalities are used in image analysis. One, cytomorphometry, measures geometric relationships, such as nuclear dimensions, chromatin texture, and nucleolar size, shape, and position. The other, cytophotometry, measures cell and nuclear optical density at various wave lengths after staining with different dyes. Since there exist innumerable tissue surrogate biomarker assays which depend on the amount of chromogen fixed to a cell or nucleus by chemical, antibody, or cDNA probes that are visualized by a second, chromogen-generating molecule (*e.g.*, a fluorescent dye or horseradish peroxidase), the range of applications of image cytophotometry is very wide. Table I outlines the different categories of analysis which may be performed on histological sections with cytomorphometry and cytophotometry.

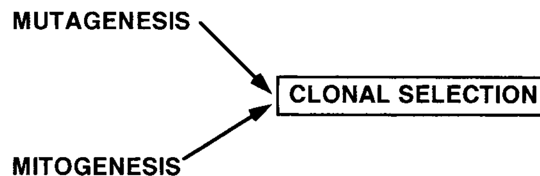

Figure 3

TABLE I. Computerized Image (Static) Cytometry: Categories of Analysis

CYTOMORPHOMETRY	
Nuclear morphometry	**References**
Size (nuclear area)	[17–19]
Shape (roundness factor)	[20]
Texture (chromocenters: number, size, shape, postion)	[21]
Nucleoli (number, size, shape, position)	[22]
CYTOPHOTOMETRY	
Chemical probes	
DNA (Feulgen stain)	[23–26]
Fluorescence reagents	[27]
Enzyme reagents	[28,29]
Immunochemical (antibody) probes	
With fluorescent markers	[31]
With enzyme markers (*e.g.*, peroxidase)	[32,33]
Hybridochemical (nucleic acid) probes	
With fluorescent markers	[34]
With enzyme markers	[35]

THE USE OF COMPUTERIZED IMAGE ANALYSIS TO ASSAY SEBs

To avoid the unacceptable cost in dollars, time, and scale of effort required to conduct Phase II clinical trials using cancer incidence reduction as the endpoint, the Division of Cancer Prevention and Control (DCPC), National Cancer Institute, has implemented a program to identify and develop SEBs which can be modulated by a chemopreventive agent in a way that predicts a reduction in cancer incidence.

The seven morphological changes of dysplasia discussed previously are components of the early neoplastic process and are completely embodied in the causal pathway to invasive cancer. When analyzed individually by computerized cytometry, they form a battery of valid SEBs which closely correlate with cancer incidence reduction.

Table II illustrates how each of the seven criteria of dysplasia may be analyzed by computerized image cytometry as an independent SEB. In addition to a cellular parameter being expressed by the mean $\pm$ a coefficient of variation (CV), each of the parameters may also be expressed either as a frequency distribution or as the fraction of cells which exhibit an optical density value above a given cutoff value set by the observer. The observer may vary the cutoff

TABLE II. SEBs Derived From Individual Criterion of Dysplasia Measured by Computer-assisted Cytometry

Pathologic Criterion of Dysplasia	Morpho- or Photometric Criterion of Dysplasia	Parameter Measured[a]
↑ Nuclear size	↑ Nuclear area (A, in μm)	↑ mean A ± CV
Altered nuclear shape	↑ Shape factor (F^b)	↑ mean F ± CV
↑ Nuclear stain uptake	↑ O.D. of Feulgen stained nuclei	↑ mean O.D. ± CV
↑ Nuclear pleomorphism	↑ CV of area, shape, and stain uptake	↑ mean CV ± (CV′)[c]
↑ Mitoses	↑ S-phase fraction (SPF)	↑ mean SPF ± CV
↑ Mitoses	↑ O.D. of Ki-67, PCNA nuclear stains	↑ mean O.D. ± CV
Abnormal mitoses	DNA aneuploidy by O.D. of Feulgen-stained nuclei	↑ Fraction of O.D. >2N
Abnormal or absent differentiation	Abnormal morphometry	↓ Differentiation markers

[a]Modulation of the parameter by a chemopreventive agent is indicated by a return of the parameter toward normal values.

[b]Shape Factor $F = A_p/A_p$, where a_p = **area** of a circle calculated from the **perimeter** of the nucleus, and A_d = **area** of a circle calculated from the mean **diameter** of the same nucleus. ↑ = increased; ↓ = decreased.

CV = coefficient of variance

[c](CV′) = CV of the mean CV

O.D. = optical density

Each of the diagnostic criteria of dysplasia used by the microscopist are shown in the left hand column. In the middle column, the equivalent parameter measured by computerized image cytometry is shown for each of these criteria. Modulation of a given parameter by a test chemopreventive agent would be shown by normalization toward normal values of the changes shown in the right hand column.

value to provide maximum separation of test versus control cell signals.

As part of its SEB program, the DCPC has also developed a battery of animal models which use dysplasia as the endpoint. These models are summarized in Table III. In addition, the DCPC has also implemented a program of short-term Phase II clinical trials which use dysplasia-based SEBs. The nature of these trials is outlined in Table IV which also shows a number of molecular biomarkers being evaluated concurrently with dysplasia in the different organ epithelia. It is noteworthy that the molecular biomarker shown at the bottom of the table, decreased mitochondrial cytochrome oxidase type III (mt COX III), occurs in microscopically normal colonic mucosa and therefore qualifies as a predysplastic SEB. Mt COX III occurs uniformly throughout the colonic epithelium in patients with familial polyposis and is therefore uniform-

TABLE III. SEB Animal Models

Organ	Animal	Carcinogen	Gross Lesion	Micro Lesion
Skin	SENCAR Mouse	DMBA	Papilloma	Dysplasia
Cheek pouch	Hamster	B(*a*)P	Leukoplakia	Dysplasia
Breast	Rat	MNU, DMBA	Tumor	Dysplasia
Trachea	Hamster	MNU	None	Dysplasia
Colon	Rat	AOM	Aberrant crypts	Dysplasia*
Bladder	Mouse	OH-BBN	None	Dysplasia

*A fraction of aberrant crypts develop dysplasia at a later timepoint.

ly accessible to biopsy, in contrast to the dysplasia-based SEBs which have a focal distribution that makes them too difficult to biopsy.

As part of the development of SEB assays, it is important to also develop procedures for their quality control, including control of variation in the tissue sample, the assay protocol, the instrumentation used, and both intra- and inter-observer variation. If an SEB assay is being performed in several laboratories, a testing program should be established by a central facility (which would distribute a battery of "unknown" test materials and analyze the results returned from the different laboratories). Only in this way will SEB assays of sufficient reliability be made available for use in clinical trial protocols monitored by the Food and Drug Administration. Finally, information should be obtained on the precision and sensitivity of the dose-response relationship, *i.e.*, between a known chemopreventive agent and its ability to modulate the SEB. Knowing whether the dose-response relationship is linear, sigmoid, hyperbolic with plateau, or other could provide leads to understanding the underlying mechanisms involved.

Better quality control of SEBs is especially needed in cases where the tissue sample is converted to a cell-free homogenate. Such assays need to be checked for variation in the number of different cell types in the original tissue sample, including normal and neoplastic epithelial cells, stromal fibroblasts and vascular endothelial cells, and many types of inflammatory cells, as well as fat, nerve, and muscle cells. Better quality control is also needed in assays which track changes at the nucleic acid level as related to oncogene amplification or mutation. Some changes may be estimated from the comparative density of bands on gels (nucleic acid or protein) developed from cell-free homogenates.

DISCUSSION

Three important concepts are highlighted here. First, the rate of progression of intraepithelial neoplasia is driven by chronic, simultaneous exposure of the epithelium to mutagens and mitogens, coming either directly from the atmosphere (impacting cutaneous and respiratory epithelia), indirectly from the diet (impacting enteric and urogenital epithelia), or from endogenous sources (*e.g.*, intracellular pro-oxidants, hormones, or growth factors). Consequently, the pace of intraepithelial neoplasia may be slowed or stopped by the continuous administration of chemopreventive drugs which block the action of these mutagens and mitogens.

Second, each of seven established diagnostic criteria of intraepithelial neoplasia (dysplasia), when assayed by the sensitive, precise, and objective technology of computerized image (static) cytometry, may be used individually as an SEB for cancer incidence reduction. Used

TABLE IV. Short Term Phase II Trials Using Dysplasia as a Surrogate Endpoint Biomarker

Organ	Chemopreventive Agent	Gross Lesion	Microscopic SEB	Molecular SEB
Skin	4-HPR	Actinic keratosis	Dysplasia	PCNA, RAR
Oral	4-HPR	Leukoplakia	Dysplasia	PCNA, EGF-R, RAR, TGF, involucrin
Lung	4-HPR	Chronic smoker's lung	Dysplasia	PCNA, EGF-R, p53, polysomy
Bladder	DFMO	Focus of redness	Dysplasia	ODC, EGF-R, Le^x
Colon	Ca^{++}	Adenomatous polyp	Dysplasia	TThy, BrdU, $extLe^x$, keratins, integrins
Cervix	4-HPR	Mosaicism, punctation	Dysplasia	DNA aneuploidy, PCNA, *ras*

Predysplastic Marker

Organ	Chemopreventive Agent	Microscopic Lesion	Molecular Lesion	Probe
Colon	Ca^{++}	None	↓ mt COX III	cDNA

PCNA: proliferating cell nuclear antigen; RAR: retinoic acid receptor; EGF-R: epidermal growth factor receptor; TGF: transforming growth factor; ODC: ornithine decarboxylase activity; Le^x: Lewis x antigen; $extLe^x$:extended Lewis x antigen; TThy: tritiated thymidinne uptake; BrdU: bromdeoxyuridine uptake; K-19: K-19 keratin antigen; mt COX III: mitochondrial cytochrome oxidase type III; MoAb: monoclonal antibody

together as a battery, these dysplasia-based markers constitute a highly valid and powerful SEB.

Third, the use of computerized image analysis of dysplasia-based SEBs solves the problem of the forbidding costs associated with using cancer incidence reduction endpoints in Phase II clinical trials of candidate chemopreventive drugs. Currently, clinical trial costs are large, not only in terms of dollars (millions), but in scale (thousands of subjects), and especially in the length of time required (frequently over five years). With computerized analysis of dysplasia-based SEBs, Phase II trials may become short-term (6–12 months), small scale (100–200 subjects), and lower cost (under one million dollars). In addition, the technology associated with dysplasia-based cytometric assays will provide the basis for developing other surrogate biomarkers at the molecular level and at the earlier, predysplastic stages of intraepithelial neoplasia.

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