Determination of Biomarkers for Intermediate End Points in Chemoprevention Trials

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Abstract

Renewed interest is being directed toward chemoprevention as a means of reducing cancer mortality. To overcome the inherent problems associated with using cancer development as a study end point, there has recently been a great surge of interest in defining the biomarkers associated with specific stages of the carcinogenic process as intermediate end points. We have detailed the evidence supporting the concept of field cancerization, a concept of general importance that is probably applicable to carcinogenesis and chemoprevention at many organ sites in humans, and presented results of tests of the potentially useful biomarkers proliferating cell nuclear antigen and blood group antigen. Because microassay techniques are more readily applicable to small biopsy samples, further expansion of these studies and exploration of panels of additional biomarkers are expected to generate exciting results in the field of chemoprevention.

Introduction

Epithelial cancer of the aerodigestive tract is an increasingly important public health problem throughout the world. Despite advances in surgery and radiotherapy, as well as in chemotherapy and supportive care, the 5-year survival rate has improved only marginally during the past 2 decades. Clearly, new research directions are needed. Renewed interest is being directed toward chemoprevention as a means of reducing the cancer mortality (1–3). Unfortunately, studies of chemopreventive approaches have been hampered by serious feasibility problems; development of cancer as the study end point requires observation of many subjects over a longer period than in standard phase III clinical trials. Therefore, there has recently been a great surge of interest in defining the biomarkers associated with specific stages of the carcinogenic process as intermediate end points (4, 5).

Two fundamental concepts are used to describe the process of carcinogenesis in the aerodigestive tract. First, the concept of "field cancerization," which was first introduced by Slaughter et al. in 1953 (6), hypothesizes that the whole field of tissue is exposed to the carcinogenic insult (e.g., cigarette smoke) and is at increased risk for developing cancer. In the case of aerodigestive tract cancers, this notion is supported clinically by the frequent association of tumors with premalignant lesions in the field at risk [e.g., oral leukoplakia (7) or bronchial metaplasia and/or dysplasia (8)] and by the synchronous or metachronous development of multiple primary tumors (9).

A second concept describing tumorigenesis in general is that of the multistep carcinogenic process (10). The driving force behind this process is thought to be genetic damage caused by continuous exposure to carcinogens, as evidenced by an increased frequency of micronuclei in the high risk tissue and in premalignant lesions (11), and its accumulation in the target tissue. Hypothetically, the greater the degree of genetic damage in the premalignant lesions, the greater the risk for cancer development. Moreover, these genetic alterations then give rise to phenotypic alterations in the tissues, such as dysregulation of the proliferation and differentiation pathway. Histologically, this is visualized as a transition from normal epithelium to hyperplasia to metaplasia/dysplasia and then to frank malignancy.

Selection of Candidate Biomarkers

Elegant models worked out by many investigators support the multistep nature of the carcinogenic process (12, 13). However, because biomarker research in chemoprevention has started recently, no individual biomarker or pattern of multiple biomarker expression has been validated recently as a useful intermediate end point. In our previous report describing the feasibility of biomarker studies in chemoprevention trials (4), we set the following criteria for the selection of candidate biomarkers: (a) differential expression in normal and high-risk tissue, (b) ability to be analyzed in small tissue specimens, (c) correlation of quantitative degree or pattern with the stage of carcinogenesis, and (d) preclinical or early clinical data supporting modulation by the study agent.

As a strategy to identify potential biomarkers expressed in premalignant lesions and in tissues at high risk but not in normal cells, we first focused on the markers found in the tumor cells (simply because of the easy accessibility of such specimens) and then examined expression of those markers in the premalignant lesions. Two chemoprevention trials (14, 15) provided access to the premalignant lesions oral leukoplakia and bronchial metaplasia. We are evaluating two general classes of biomarkers, genetic and phenotypic (e.g., proliferation and differentiation markers). The purpose of this communication is to present a review of preliminary data, which suggest that this approach is technically feasible.

Genetic Markers: Further Evidence for Field Cancerization

The whole carcinogen-exposed field presumably accumulates genetic damage; however, only a few malignant foci actually develop. It is hypothesized that only those cells that accumulate the “right” hits progress towards frank malignancy (16). The identity of these important genetic events is unclear. Nevertheless, complex chromosomal abnormalities are consistently found not only in the tumor cells (17–19) but also in the “normal” but exposed nonmalignant lung tissue samples from patients with lung cancer (17, 19). Unfortunately, most cytogenetic studies of aerodigestive tract cancers have been performed on cell lines or after short term culture, which might allow the development of in vitro artifacts (e.g., preferential outgrowth or lack of growth of subpopulations or new changes occurring ex vivo).

To overcome some of the problems of solid tumor cytoge-
netics, we previously examined both primary lung tumor and “normal” lung tissue obtained from the same patients at the time of tumor resection (20, 21), using the premature chromosome condensation technique. The premature chromosome condensation technique is based on the original observation of Johnson and Rao (22) that, when mitotic cells are fused with interphase cells, the nucleus of the interphase cell undergoes an immediate prophase-like reaction, such that the nuclear envelope is disassembled and the chromatin is condensed into chromosomes. Since the chromosomes of the target cell population can be visualized without cell culture, this technique overcomes the problem of culture artifacts and also permits the cytogenetic analysis of slowly proliferating or nonproliferating cell populations (20).

Of the seven lung tumors evaluated in those studies (20, 21), two showed predominantly hypodiploid populations, one both a hypodiploid and a hypotetraploid population, three hyperdiploid, and one a near-diploid peak. Fusions with “normal” lung cells from the same patients yielded from 40 to 70 chromosomes/cell. As expected, cells with 46 chromosomes were also present in all cases, suggesting a heterogeneous mixture of cells from the normal lung. Most interesting, in all but one of the normal lung samples, the distribution of chromosomes numbers per cell resembled that in the corresponding tumor specimens (21). Successful banding analyses showed that the tumor cells and normal cells shared some cytogenetic abnormalities. As might be expected from genetic evolution, the tumor cells also contained chromosomal anomalies not found in the normal cell. However, the normal cells also contained certain chromosome changes not observed in the tumor. These results strongly support the notions of field cancerization and multistep carcinogenesis and suggest that most of the complex cytogenetic abnormalities found in aerodigestive tract tumors represent chromosomal damages that accumulated before malignant transformation rather than secondary changes occurring after the primary event that caused frank malignancy.

More convincingly, further evidence was generated by applying a newly developed in situ “chromosome painting” technique, a modification of the fluorescent in situ hybridization technique (23), to the paraffin-embedded tissue samples. Briefly, using biotin-labeled chromosome-specific repetitive DNA probes (Oncor, Gaithersburg, MD) and the ABC immunoperoxidase staining technique, we were able to demonstrate chromosomal alterations in the cells within the histologically defined premalignant oral lesions.

**Detection of Cellular Proliferation**

**General.** Since an important component of carcinogenesis is unregulated growth, we adopted techniques for detecting cell proliferation in tissues at risk. We employed antibodies against the proliferation markers topoisomerase II, DNA polymerase-α, Ki-67, and PCNA and found PCNA to be quite useful as a marker for proliferating cells, even in the routinely processed, formalin-fixed, paraffin-embedded tissue samples (15, 24). PCNA, also called cyclin, is a 36-kDa intranuclear polypeptide (25, 26). It has been shown that PCNA is an auxiliary protein of DNA polymerase-δ (27) and that it plays a critical role in cell proliferation (28). Its expression fluctuates dramatically during the cell cycle (29, 30), and it appears in trace amounts in the G1 phase, increases to its maximum in the S phase, and slowly declines thereafter. Recently, data on cell kinetics obtained by immunostaining for PCNA in paraffin-embedded tissue samples were reported (31, 32).

To better understand the relationship of PCNA expression in tissue and the proliferative status, we, in collaboration with Dr. Nick Terry of the Department of Experimental Radiotherapy at our institution, examined blocks of paraffin-embedded specimens of head and neck and colorectal cancers resected from patients who had received a pulse iv infusion of BrdUrd just before surgery. Adjacent tumor sections were analyzed for PCNA expression and for incorporated BrdUrd (using anti-PCNA antibody and anti-BrdUrd antibody, respectively). Regions of tumors that were high in PCNA-positive cells were also high in BrdUrd uptake and vice versa. In all cases, the proportion of PCNA-positive cells was higher than the proportion of BrdUrd-positive cells. These results are expected, since PCNA is found to be expressed in most proliferating cells in G1, S, and G2, whereas BrdUrd only marks the cells in S phase.

Tumors. Since PCNA expression might be dysregulated in tumors, we examined paraffin blocks of tumor specimens from 107 patients who underwent surgical resection for non-small cell lung cancer (24). Of these, 61 patients had adenocarcinoma, 37 had squamous cell carcinoma, and 9 presented with large cell carcinoma. Squamous cell carcinoma showed the highest proliferative activity, with a median of 40% PCNA-positive cells (range, 2–90%), compared with 5% PCNA-positive cells for adenocarcinoma (range, 0–70%) and 15% for large cell carcinoma (range, 3–80%). There was an inverse relationship between histological grade and the proportion of PCNA-positive cells. Normal bronchial epithelium exhibited none to very few PCNA-positive cells, and those were confined to the basal layer. The PCNA-positive fraction became progressively higher in areas of squamous metaplasia and carcinoma in situ.

**Premalignant Lesions.** As a part of an ongoing chemoprevention trial, where chronic smokers (≥15 pack-year smoking) were screened for squamous metaplasia before being randomized to receive either 13-cis-retinoic acid or placebo, we examined PCNA expression in bronchial biopsy sections obtained from six standardized sites at the major bronchial bifurcations (15). So far, 165 samples have been evaluated for histological status as well as PCNA expression. Among the 81 biopsy specimens showing histologically normal epithelium, only 12% had >1% PCNA-positive cells, and no specimen had >5% PCNA-positive cells. In contrast, 37% (19 of 52) of the specimens showing hyperplasia and 50% (10 of 20) of the sites showing squamous metaplasia had >1% PCNA-positive cells. Among those biopsy specimens showing squamous metaplasia with dysplasia, 58% (7 of 12) had >1% PCNA-positive cells and, of these seven, four specimens had >5% PCNA-positive cells. These results suggest a significant correlation between increase in proliferative activity and histological progression from normal to hyperplasia to squamous metaplasia/dysplasia.

The greatest variance in degrees and patterns of PCNA expression (e.g., basal to suprabasal extension) occurred in the subgroup of high-risk tissues that showed metaplasia/dysplasia, suggesting dysregulated proliferation and possibly different risks of malignant transformation. In a study of esophageal premalignancy by Yang et al. (33) and a study of subjects at high risk for colon cancer by Lipkin et al. (34, 35), the patterns of expression of the proliferation marker titrated thymidine incorporation were similar to the patterns of PCNA expression we observed in the lung. Those pilot studies also have suggested...

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*The abbreviations used are: PCNA, proliferating cell nuclear antigen; BrdUrd, bromodeoxyuridine; EGFR, epidermal growth factor receptor.*

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* Unpublished data.
that this marker is suppressed by chemopreventive agents. Although it is too early to evaluate their role as biomarkers of intermediate end points, our results and others' suggest that proliferation markers are promising for this role.

**Detection of Differentiation Marker ABH Blood Group Antigen**

**General.** Blood group antigen is a normal differentiation marker that is lost during the multistep carcinogenesis process. Its expression in relation to cancer development and cellular differentiation and maturation has recently been the subject of renewed interest (36, 37). ABH blood group antigens are normally found on RBC and in a variety of epithelial cells, and their antigenic determinants are carbohydrate side chains of glycoproteins and glycolipids. Altered expression of ABH blood group antigens during malignant transformation and tumor progression has been reported (38-40). Recently, in examining the expression of EGFR in paraffin-embedded samples of nonsmall cell lung cancer, we found that immunostaining with the anti-EGFR monoclonal antibody 29.1 was associated with a favorable clinical outcome after surgical resection (41). Because this anti-EGFR antibody has been found to cross-react with the blood group antigen A epitope (42), we expanded the study to determine whether blood group antigen expression by nonsmall cell lung cancer cells has the same prognostic significance. Since tissue expression of blood group antigens is dependent on blood type, we studied patient survival within the context of ABO blood group and found that tumor cell expression of blood group antigen A, but not of blood group antigen B or H, was an important favorable prognostic factor after surgery in patients with non-small cell lung cancer (43).

**Oral Premalignancy.** Since retinoic acid is known to restore the normal differentiation and maturation process (44), we examined blood group antigen A expression in routinely processed oral leukoplakia lesions and compared the results obtained before and after 13-cis-retinoic acid treatment (1.5 mg/kg daily for 3 months) (45). Of the first 55 patients registered, 28 were of blood type A or AB and are the subject of this analysis. Of 37 lesions examined at baseline (9 patients had multiple site involvement), blood group antigen A expression was negative in 14 (38%), focally positive in 7 (19%), and diffusely positive in 12 (32%), while 4 (11%) of the lesions had mosaic patterns. Of the 25 patients who had tissue samples reexamined after 3 months of 13-cis-retinoic acid treatment, 9 (36%) patients had an increase in blood group antigen A expression and 13 (52%) had no change in its expression, as compared to the base line, while 2 (8%) had a decrease and 1 (4%) had mixed results (i.e., one lesion showed an increase but the other a decrease). Although preliminary, these results are interesting not only because they confirm previous observations of altered blood group antigen expression in oral premalignant lesions (39, 40) but also because they suggest that chemopreventive agents can modulate biomarker expression.

**Bronchial Premalignancy.** To better understand the role of blood group antigen expression in lung cancer development, we examined bronchial biopsy samples from chronic smokers, as described above. In normal bronchial mucosa, only the basal layer cells were stained with ABH blood group antibodies. This is in contrast to results for the normal squamous epithelium, in which ABH blood group antigens are not expressed by basal layer cells but are expressed as the cells differentiate and mature into the suprabasal and superficial layers. Interestingly, we observed areas of loss of blood group antigen expression, which was focal in some places (restricted to a cluster of 10–15 cells) and patchy in others. Histologically, the nonstaining areas did not differ from adjacent areas that were stained.

These results, taken together with our and others' results for the oral premalignant lesions (39, 40, 45), as well as results for lung cancer (43, 46, 47), head and neck cancer (48), and transitional carcinoma of the bladder (49, 50), strongly suggest that altered blood group antigen expression is a promising biomarker of intermediate end points.

**Potential Use of Biomarkers in Chemoprevention Trials**

The goal of clinical trials of chemoprevention in the aerodigestive tract is to reduce the risk of cancer development in that field. However, as previously mentioned, the major obstacle to such trials is that the study end point (i.e., cancer development) is extremely distant. It would be useful to define intermediate end points that might reflect whether the chemopreventive agent has had an effect on the tissue at risk (4, 5) and, if so, also reflect its mechanism of action at the tissue level. Ideally, biomarkers could be used early in the study to distinguish those individuals who are responding to chemopreventive treatment from those individuals who are not responding.

In addition, these markers would allow us to better understand the pathobiology of clinical response to the chemopreventive treatment. For example, if a premalignant lesion such as oral leukoplakia were shown to exhibit trisomy 7 by in situ chromosome painting techniques and also to express high levels of EGFR (whose gene is located on chromosome 7) or other markers (e.g., blood group antigen), one might want to know whether the clinical response to the treatment is due to (a) a phenotypic reversal of the abnormal clone, (b) complete elimination of abnormal clones, or (c) regrowth of less affected clones (if two or more distinct clones are in the region). This question could be resolved by examining both genetic markers and phenotypic markers on the samples obtained before and after treatment. Finally, although strict validation of any biomarker as a true intermediate end point of cancer development may take many years of follow-up of a large number of subjects (5), currently available promising biomarkers are an important adjunct to the development of new chemopreventive agents and to the rational design of future intervention trials. Once validated, however, specific biomarkers may be used as surrogate end points, replacing cancer development in that role.

**Conclusion**

Biomarker research in chemoprevention trials is still in its infancy. Nevertheless, we have detailed the evidence supporting the concept of field cancerization, a concept of general importance that is probably applicable to carcinogenesis and chemoprevention at many other sites in humans (e.g., skin, gastrointestinal tract, and genitourinary and gynecological sites). In addition, we have presented results of tests of the potentially useful biomarkers PCNA and blood group antigen and, more important, we have demonstrated that multiple biomarker studies are now technically feasible because microassay techniques are more readily applicable to small biopsy samples. Further expansion of these studies and exploration of panels of additional biomarkers, including genes at the chromosome 3p depletion site, growth regulatory genes, and dominant and recessive oncogenes (e.g., those coding for transforming growth factor-β and EGFR, c-myc, ras, and p53), are expected to generate exciting results in the field of chemoprevention.
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