Journal of Cellular Biochemistry, Supplement 23:104-112 (1995)

Chemoprevention Trials in the Cervix: Design, Feasibility, and Recruitment

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Abstract The cervix is an ideal organ for chemoprevention studies and the study of squamous carcinogenesis. In chemoprevention trial design, four factors are important: high-risk cohorts must be identified; suitable agents must be selected; study designs should include Phase I, II, and III; and studies should include the use of surrogate endpoint biomarkers. High-risk cohorts can be selected for Phase I, II and III trials in the cervix, for example, patients with high grade lesions such as cervical intraepithelial neoplasia (CIN) grade 3 and carcinoma *in situ* (CIS). A Phase III trial might also include patients with lesions infected with oncogenic HPV types. The cervix is accessible and can be safely followed with Papanicolaou (Pap) smears and colposcopy. Suitable agents include those likely to work in squamous lesions, including retinoids, difluoromethylornithine, β-carotene, and others. In Phase I chemopreventive studies, doses are de-escalated rather than escalated, determining toxicity and optimal dose schedule. Phase II studies looking at effectiveness need placebo control groups since regression of high-risk lesions is possible. Phase III studies, now multicentric, should be carefully designed and include wide patient representation in order to evaluate the risk-benefit ratio of therapy, focusing on cancer incidence reduction. Surrogate endpoint biomarkers include quantitative histopathology, biologic measures of proliferation, regulation, differentiation, genetic instability, and fluorescence emission. Quantitative histopathologic markers include nuclear grading (*i.e.*, shape, area, optical density, texture), nuclear pleomorphism, ploidy, and nucleolar size and position. Biomarkers under study at the present time in the cervix include proliferation markers (PCNA), regulation markers (EGFR, *ras*, *myc*, p53, retinoic acid receptors, ODC, spermidine/spermine ratios), differentiation markers (involucrin, cornifin, keratins), and markers of genetic instability (chromosome polysomy). Fluorescent spectroscopy uses light to probe the biochemical properties of tissue. This technique provides an automated diagnosis in real time with comparable

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sensitivity and specificity to colposcopy and can be used to monitor lesions in chemoprevention trials. Recruitment designs for cervix studies need to include a large referral population and patients with sufficiently large lesions. Clinicians involved in such studies need to stress contraception and smoking cessation, deal with language barriers, and provide compensation for child care and parking to patients in order to increase compliance.

Key words: Chemoprevention, cervical intraepithelial neoplasia, fluorescence spectroscopy, squamous intraepithelial lesion

Sporn cautions that we are far behind the cardiovascular community in recognizing the importance of early precursor lesions as antecedents of clinically symptomatic disease. He believes cancer is a process rather than an event: "The disease process is carcinogenesis, not invasive or symptomatic cancer. Invasive and metastatic cancer are clinical and pathological end stages, at which it may be too late to prevent further progression" [1]. Chemoprevention refers to the use of chemical agents (micronutrients, pharmaceuticals) to prevent or delay the development of cancer in healthy populations. These agents, which block the initiating and promoting events of carcinogenesis, provide a tertiary preventive measure [2] and augment the preventive armamentarium, which includes avoiding carcinogens in the environment (primary prevention) and participation in screening programs (secondary prevention) [3].

Agents are classified by their mechanism of action—those that block or suppress mutation, promotion, both mutation and promotion, and those whose mechanism is unknown [4]. The Chemoprevention Branch of the Division of Cancer Prevention and Control at the National Cancer Institute has developed a program that identifies candidate drugs, characterizes their action through *in vitro* and *in vivo* animal studies, conducts pharmacokinetic and toxicological studies, and supports Phase I, II, and III testing [5]. Detailed reviews of the program and processes for drug evaluation are available [4–7].

Despite the availability of the screening Papanicolaou (Pap) smear, cervical cancer remains an important health problem in women. It is the second most common malignancy in women worldwide; incidence rates are currently increasing in American women [8]. According to Surveillance Epidemiology and End Results (SEER) estimates, 15,800 women will be diagnosed with invasive cervical cancer in 1995 (up from 15,000 in 1994 and 13,500 in 1993); 4,800 women will die from invasive cervical cancer (up from 4,600 in 1994 and 4,400 in 1993), and 65,000 women will be diagnosed with carcinoma *in situ* (up from 55,000 in 1994 and 50,000 in 1991) [8–11]. An estimated 2,500,000 women will have abnormal Pap smears which will have low-grade dysplastic lesions [12]. The exact number with high-grade abnormalities is unknown.

The cervix is a unique organ well suited to the development of chemoprevention trials. The cervix has long been considered by pathologists as a model for the progression from mildly dysplastic lesions through severely dysplastic lesions to invasive cancer. The ability of clinicians to follow cervical lesions with colposcopy and Pap smears makes such studies feasible. Biologic studies of cervical carcinogenesis will surely contribute to our understanding of the neoplastic process and hence, development of new preventive and therapeutic strategies.

DESIGN

The design of chemoprevention studies involves four elements: identifying high-risk cohorts, selecting suitable agents, including Phase I, II, and III study designs, and using surrogate endpoint biomarkers (SEBs). Theoretically, three groups of patients are eligible for such trials: those who are at high risk for cancer but without a precancerous lesion, those with a precancerous lesion, and those with a previous malignancy who are at high risk for a second primary or for recurrence. Risk profiles may be based on genetic factors, life-style, environmental exposures, a history of a precursor lesion, or some combination of these [4,8,13,14].

The pharmaceuticals under investigation have been described in detail [6,7]. Retinol and β-carotene are under investigation in the skin; 13-*cis*-retinoic acid and β-carotene in the oral cavity;

tamoxifen and *N*-(4-hydroxyphenyl)retinamide (4-HPR) in the breast; β-carotene, vitamin E, 13-*cis*-retinoic acid, and retinol in the lung; the nonsteroidal antiflammatory drugs (NSAIDs) piroxicam and sulindac, along with wheat bran, 2-difluoromethylornithine (DFMO), calcium carbonate, vitamin E, vitamin C, and β-carotene in the colon; and β-carotene, β-all-*trans*-retinoic acid, folic acid, DFMO, and 4-HPR in the cervix [7].

Chemoprevention trials have several unique features which distinguish them from therapeutic trials. These features involve several disciplines to create trials which weave the biology of carcinogenesis into the trial design [13,14]. Phase I trials in chemoprevention, like chemotherapy trials, seek to characterize the pharmacological and toxicological properties of the drug. In contrast with Phase I chemotherapy trials, chemoprevention trials are often dose de-escalating and seek the lowest dose at which biological modulation of the marker takes place. Since the drug will be used in patients who feel otherwise well, it should produce few side effects. Patients with well-defined preneoplastic lesions are desirable for Phase I trials.

Phase II chemoprevention trials, like Phase II chemotherapy trials, evaluate the effectiveness of drugs in a given organ. In contrast to Phase II chemotherapy trials, chemoprevention trials seek patients with preneoplastic lesions rather than neoplasms, and necessitate a concurrent placebo-control group because of the frequent regression observed in some preneoplastic lesions. Phase II chemoprevention trials seek to use SEBs rather than cancer development as endpoints. SEBs allow trials to be shorter, require fewer subjects, be lower in cost, use small tissue samples, and aid in learning more about the carcinogenic process [7].

Phase III trials evaluate the cost-benefit ratio of treatments in multicentric settings. In contrast to Phase III chemotherapy studies, which compare agents to standard therapies, chemoprevention studies evaluate cancer incidence reduction. Target patients for Phase III trials are at high risk of developing cancer. The trials are designed to follow patients for a sufficient period of time to detect a reduction in cancer incidence and are thus expensive, long, large (many subjects), and require expensive diagnostic tests as endpoints. Their design requires the placebo group to have sufficient patients to experience a sufficient incidence of the event of interest, namely the development of cancer [7].

Boone *et al.* [13] link the pathologic continuum to the single-continuum model of carcinogenesis advanced by Foulds, in which the term "progression" was used to describe the entire neoplastic process from the initial monoclonal focus of dysplastic cells to full-thickness involvement of grade 3 intraepithelial neoplasia. The concept of clonal evolution, in which genetically variant cells are selected for growth advantage and result in clonal expansion, explains, in part, the field cancerization process. The production of the genetically variant cells associated with clonal evolution is thought to be due to genetic instability, manifested by gene mutations, gene amplifications, chromosomal structural rearrangements and defects, and aneuploidy. Continuous mutagenesis and mitogenesis stimulate the rate of clonal evolution and neoplastic progression. The entire field, exposed to the carcinogen, is at high risk for genetic instability. Examples of field cancerization and exposure to carcinogens include skin exposure to the sun, aerodigestive tract exposure to cigarette smoke, and female genital tract exposure to human papillomavirus (HPV). The morphological criteria of intraepithelial neoplasia are increased nuclear size, altered nuclear shape, increased nuclear stain uptake, nuclear pleomorphism, increased mitoses, abnormal mitoses, and disordered or absent maturation [15, 16].

Before SEBs are deemed useful in chemoprevention trials, several questions must be answered. These are well outlined by Kelloff *et al.* [2,5–7]. Is the SEB differentially expressed in normal and high-risk tissue? At what stage of carcinogenesis does the marker appear? Do the marker and its assay provide acceptable sensitivity, specificity, and accuracy? How easily can the marker be measured? Can the marker be modulated by chemoprevention agents? Does modulation of the SEB correlate with a decrease in cancer rate [8]?

Examples of SEBs include cytological and histological markers, proliferation markers, differentiation markers, regulation markers, markers of genetic instability, and fluorescence spectroscopic emission. Histologic progression and regression can now be objectively measured using computer-assisted image analysis [13]. The measurable morphological criteria of intraepithelial neo-

plasia are increased nuclear size (increased nuclear area), altered nuclear shape (increased nuclear shape factor), increased nuclear stain uptake (increased optical density of Feulgen-stained nuclei), nuclear pleomorphism (increased coefficient of variation for area, shape, and stain uptake), increased mitoses (increased S-phase fraction, increased PCNA staining), abnormal mitoses (DNA aneuploidy by optical density of Feulgen-stained nuclei), and disordered or absent maturation (increased deregulation markers, decreased differentiation markers, presence of chromosome polysomy) [13].

FEASIBILITY

Cervical intraepithelial neoplasia (CIN) is defined as "the spectrum of intraepithelial changes beginning as a generally well-differentiated neoplasm, traditionally classified as mild dysplasia, and ending with invasive carcinoma" [17]. These changes, confined to the squamous epithelium above the basement membrane, include nuclear pleomorphism, loss of polarity, presence of abnormal mitoses, and lack of differentiation as cells progress from the basement membrane to the surface epithelium [17].

Richart coined the term CIN and devised a grading system in the 1960s [18]. CIN lesions were graded 1–3 based on the amount of undifferentiated cells present from the basement membrane; when up to one-third of the distance from basement membrane to surface was involved, the lesions were designated grade 1; when more than one-third and less than or equal to two-thirds was involved, grade 2; and when more than two-thirds was involved, grade 3. Full-thickness involvement, previously carcinoma *in situ* (CIS), in the Richart classification was called grade 3 CIN. The terminology defining CIN is changing [17].

The National Cancer Institute convened a panel to address the issue of Pap smear classification in 1988. The resulting classification is known as the Bethesda classification [19]. The panel's goal was to define a uniform terminology for smear reading, standards for adequacy of the smear, and guidelines for tying the smear result to clinical management of the patient. The underlying philosophy of the Bethesda classification is that it is difficult to distinguish between lesions with HPV and CIN 1, so they are combined into one category called low-grade squamous intraepithelial lesion (LGSIL). Similarly, CIN 2 and 3 lesions are placed in a category called high-grade squamous intraepithelial lesion (HGSIL). These terms, although designed for use by the cytology community, have been adapted for use in histopathology. Many institutions now report lesions in the following manner: HPV/LGSIL, CIN 1/LGSIL, CIN 2/HGSIL, and CIN 3/LGSIL. The European community has not embraced the Bethesda classification. Many among them feel the lesions should be grouped differently, with HPV in a separate category from CIN 1 and 2, and these in a different category from CIN 3 and CIS [personal observation].

The cervix is a nearly perfect organ for chemoprevention studies because of its accessibility. CIN is an excellent example of a histologic model of progression to cancer. The cervix can be followed cytologically with the Pap smear and visually with colposcopy and colposcopically directed biopsies. Lesions must be large enough to withstand multiple biopsies. Additionally, since 10–20% of patients with CIN have associated vaginal intraepithelial neoplasia (VAIN) and vulvar intraepithelial neoplasia (VIN), systemic therapy is warranted.

Patients are evaluated using colposcopy, the use of a magnifying lens to view the cervix after the placement of 3–6% acetic acid. The goal in evaluating the lesions is to exclude malignancy. The colposcopic impression is used to predict the degree of lesion present. In a large review of articles by expert colposcopists, the sensitivity of colposcopy was 94%, the specificity 51%, the positive predictive value 83%, and the negative predictive value 74%. Low-grade lesions were the ones most often misclassified. In these same series, 52% of lesions were incorrectly classified, which would have led to 38% of patients being treated incorrectly [20]. Several excellent atlases describe colposcopic abnormalities. Generally, acetowhite epithelium is considered abnormal, and as vascular atypias (punctuation, mosaicism, and atypical vessels) appear and become irregular, the suspicion of invasion increases. Although it is unknown whether the lesion itself or the infection with HPV causes angiogenesis, several studies have confirmed the usefulness of vascular atypia in predicting the severity of the lesion. Atypical vessels are the hallmark of invasive cancer.

Two groups at high risk of cervical cancer are easily identified—those with high-grade lesions and those with oncogenic-type HPV. There have been several prospective follow-up studies of the natural history of the disease, in which patients with CIN and CIS were enrolled and followed with cytology, colposcopic observation, and biopsy only. Most of these did not include HPV typing [21]. These studies included 6,086 patients with CIN and 353 patients with CIS. Some of the series, particularly those with patients with CIS, followed patients for 20 years. Of patients with untreated CIN grades 1–3, 14% progressed to CIS and 1.4% to invasive cancer. Of patients with untreated CIS, 36% progressed to invasive cancer. CIS lesions are clearly much more likely to go on to invasive cancer than CIN lesions, and must be evaluated and treated more aggressively than lower grade lesions. Thus, patients with high-grade lesions are a suitable high-risk cohort for chemoprevention studies [21].

Another high-risk cohort for chemoprevention studies is patients infected with HPV types associated with a high risk for progression to invasion based on their association with high-grade lesions and cancers. Epidemiologic evidence has long suggested that cervical neoplasia behaved like a sexually transmitted disease; laboratory and epidemiologic research has focused on the etiologic role of some types of HPV in the pathogenesis of cervical neoplasias [22–24]. HPV DNA has been detected in more than 70% of specimens from women with Pap smears showing definite cervical neoplasia and cancer, in 24% of women with Pap smears showing borderline atypia, and in 6% of women with normal Pap smears. HPV, in epidemiologic terms, is thought to be necessary but not sufficient in the causal pathway for cervical carcinogenesis. The most common HPV types detected in cervical lesions are those classified as high-risk (16, 18, 45, and 56) and intermediate-risk (31, 33, 35, 51, 52, and 58) [25]. The most prevalent HPV type is HPV 16, which is detected in approximately 90% of HGSIL and cancer [25]. Results from recently published epidemiologic studies support an association between cervical neoplasia and HPV, that is markedly stronger with HPV type 16 [26,27]. Patients infected with oncogenic-type HPVs may be suitable for Phase III chemoprevention trials once HPV-testing issues are settled and natural history studies are complete.

Promising chemopreventive agents to be investigated in the cervix are 4-HPR, DFMO, NSAIDs, β-carotene, and folate [7]. 4-HPR is a member of the retinoid group, which includes vitamin A and its natural and synthetic analogs. Vitamin A is necessary for the normal growth and differentiation of epithelial tissues. The cellular and molecular mechanisms by which retinoids act are mediated by retinoic acid receptors. The retinoids are toxic; an *N*-substituted carboxyamide group in place of the terminal carboxyl group is believed to account for the decreased toxicity seen with 4-HPR. DFMO is an irreversible inhibitor of ornithine decarboxlase (ODC), a key enzyme in the biosynthesis of polyamines, which have been shown to be essential to maintain cell growth and transformation. Tumor formation in experimental animals is prevented by ODC inhibitors such as DFMO. NSAIDs are chemopreventive and are thought to play a role in the control of neoplastic and non-neoplastic cell proliferation and immune function through the inhibition of cyclooxygenase activity and, ultimately, endogenous prostaglandin biosynthesis. β-Carotene, the most active and common carotene found in the diet, is a remarkably potent source of vitamin A. It is metabolized to retinaldehyde and then converted to retinol. It is thought to be a promising agent based on data from nutritional studies demonstrating β-carotene deficiencies in CIN patients compared to controls. Folate, specifically red blood cell folate, similar to β-carotene, has been shown to be deficient in CIN patients compared to controls. Thus, chemopreventive supplement studies with β-carotene and folate are being performed.

Several chemoprevention trials have been successfully conducted for the cervix. All trials have been in patients with CIN lesions. There have been no studies of women at risk for CIN but without lesions. Similarly, there have been no studies to prevent second primaries in women with invasive cervical cancer. Phase I and II studies of the cervix are under way or were recently completed using 4-HPR, DFMO, β-carotene, folate, and topical β-all-*trans*-retinoic acid in high-grade CIN lesions. Three principal groups have published chemopreventive studies in the past: Romney *et al.* [28], Butterworth *et al.* [29,30]; and Surwit *et al.*, Meyskens *et al.*, and Weiner *et al.* [31–34].

Romney *et al.* [28] reported on a Phase I–II

trial using retinal acetate gel topically in patients with CIN 1–2; the trial showed that high compliance could be achieved and determined the approximate dose for a Phase III trial [28]. There is no published report of the Phase III trial. This group is currently conducting a Phase II trial of β-carotene at 30 mg/day [5]. Results have not yet been published.

Butterworth *et al.* [30] published an update of a randomized trial in which patients with CIN 1 and 2 lesions were treated with folate (10 mg) or vitamin C (10 mg) as a placebo, each for 90 days. There were no statistically significant differences in the two groups in regression of lesions in the 177 evaluable patients. Folate supplementation was thought to be a good choice for a chemopreventive based on studies showing decreased red blood cell folate levels in women with CIN [29]. A second study of folate supplementation by Childers has had similarly negative results [35].

Phase I and II trials by Surwit *et al.* [31], Meyskens *et al.* [32,33] and Weiner *et al.* [34] demonstrated that all-*trans*-retinoic acid could be safely delivered topically to the cervix, and that it was more likely to achieve a response at a dose of 0.37% than at lower doses. Meyskens [33] recently reported the results of the randomized Phase III trial of 0.37% all-*trans*-retinoic acid in patients with CIN 2 and 3 lesions, in 151 and 150 patients, respectively. Patients with CIS were excluded from the study. Patients were initially treated with 0.37% retinoic acid daily for four days, then treated for two days each at three- and six-month follow-up visits. Patients were seen for Pap smears and colposcopy at 9, 12, 15, 21, and 27 months. Biopsies were performed at the 15-month visit. Losses to follow-up were large. Of 151 patients randomized to placebo, 81 patients were evaluated at 15 months and 25 patients at 27 months. Of 150 patients randomized to retinoic acid, 88 patients were seen at 15 months and 21 patients at 27 months. There was a statistically significant regression in the CIN 2 lesions in treated patients compared to placebo, but not in the CIN 3 lesions in treated patients [33]. Sporn and Roberts [36] speculated in an editorial that the reason CIN 2 lesions responded and CIN 3 did not is that lesions farther along the path toward neoplasia may be harder to regress, requiring higher doses, longer administration, systemic administration, or two agents instead of one.

A. Manetta and M. Berman [37] have undertaken a study of β-carotene in patients with CIN. No results are yet published.

Studies underway at our institution include those with DFMO and 4-HPR. Both medications are given orally, and thus the effects are systemic. The female genital tract, like the aerodigestive tract, is subject to the field cancerization process. Women with CIN are highly likely to harbor VAIN and VIN. Systemic therapies are logical since cancer and pre-cancers are systemic processes. The designs underway include the incorporation of surrogate endpoint biomarkers, including quantitative histopathology, biologic measures of carcinogenesis (measures of proliferation, regulation, differentiation, and genetic instability), and fluorescence spectroscopic emission. Quantitative histopathologic measurements are being made using the CAS-200 and the Cytosavant® on tissue sections and cytologic smears [38,39]. Results of quantitative pathologic measurements will be correlated with SEBs and fluorescence spectroscopy. Preliminary data on archival specimens show statistically significant increases in nuclear size and optical density as lesions progress from low- to high-grade intraepithelial neoplasia. Fluorescence spectra also correlate highly with optical density.

SEBs under study in our institution include proliferation markers (PCNA), regulation markers (EGFR, *ras*, *myc*, p53, retinoic acid receptors, ODC, spermidine/spermine ratios), differentiation markers (involucrin, cornifin, keratins), and genetic instability markers (chromosome polysomy). Preliminary work on archival specimens demonstrated statistically significant increases of PCNA, EGFR, and *ras* as lesions progressed from low- to high-grade, and statistically significant decreases of involucrin and cornifin as lesions progressed from low- to high-grade [21].

Fluorescence spectroscopy uses light to probe the biochemical properties of tissue. Tissue is illuminated with monochromatic light via optical fibers and the resulting fluorescence intensity, as a function of wavelength, is measured quantitatively. The fluorescence spectra contain information about the presence of tissue fluorophores (NADH, FADH, elastin, collagen) and absorbers (hemoglobin). The penetration of ultraviolet and visible excitation wavelengths of light is limited to several hundred microns and is thus well-suited to the detection and diagnosis of intraepi-

thelial lesions. This technique provides an automated diagnosis in real time with little or no training of the provider performing the measurement. *In vitro* and *in vivo* work in cervical epithelium demonstrates that measurements at 337, 380, and 460 nm excitation wavelength can be used to develop a diagnostic algorithm with comparable sensitivity, superior specificity, positive predictive value, and negative predictive value to that of colposcopy [40–43]. The value of this technique in chemoprevention studies is twofold: first, lesions not visible during examination with the naked eye or during white light scoping can be targeted for biopsy; second, small lesions that might be affected by biopsy can be followed without biopsy.

RECRUITMENT

Many issues arose in designing our trials in the cervix. For example, it is necessary to see a sufficiently large referral base to collect patients with large, high-grade lesions. The patients must be willing to use contraception, since the drugs are teratogenic. Many of the patients are smokers. In our tri-ethnic population, language barriers must be addressed in study explanation, consent, and clinical care. Compensation for child care and parking has been greatly appreciated by patients.

We have found that patients with a family history of cancer, those unafraid of "research", and those who grasp the concepts of chemoprevention are more willing to participate than those with negative family histories, fear of research, and fear of the process. Thus far, our recruitment is over 50% of those with eligible lesions, and the compliance with visits in our Phase I trial is 100%. Adherence to drug dosage has not yet been assessed. Barriers to participation thus far have been age, fear of cancer, transportation and child care dilemmas, pregnancy, and personality disorders.

CONCLUSION

The cervix is a nearly perfect organ for chemoprevention studies because of its accessibility and ability to be followed with colposcopy and Pap smears. CIN is an excellent example of a histologic model of progression to cancer. Quantitative histopathology has already proven useful in the cervix. There are several studies of individual biologic markers of carcinogenesis, but they have not yet been linked in a meaningful way. Fluorescence spectroscopy, providing a real-time diagnosis and a view of the biochemical properties of tissue, will become an important biomarker as algorithms are developed which improve its sensitivity and specificity. Lessons learned in the cervix may well unravel some of the mystery surrounding squamous carcinogenesis and provide new targets for intervention.

ACKNOWLEDGMENT

We gratefully acknowledge support from NCI contracts NO1-CN-25433A and NO1-CN-25433B.

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