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Activity of 4-HPR in Superficial Bladder Cancer Using DNA Flow Cytometry as an Intermediate Endpoint

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Abstract The ability of the synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4-HPR) to affect the outcome of previously resected superficial bladder cancer was investigated in a pilot study using DNA content flow cytometry and conventional cytology as intermediate endpoints. Twelve patients were treated with oral 4-HPR (200 mg daily) and compared with 17 non-randomized, untreated controls. The median interval between transurethral resection and 4-HPR administration was 5.5 months (range 0–36). The median follow-up period was 12 months (range 3–31) in the 4-HPR group and 9 months (range 2–22) in the control group. The proportion of patients with DNA aneuploid stemlines in bladder-washed cells decreased from 7/12 (58%) to 5/11 (45%) in the 4-HPR group, but increased from 7/17 (41%) to 10/17 (59%) in the control group. In patients with stable diploid profiles, mean (±SE) S-phase and G2+M-phase fractions decreased in the course of retinoid treatment from basal levels of 15.2 ± 4.1% to 7.5 ± 3.3% and 10.3 ± 2.2% to 5.2 ± 0.4%, respectively. The same parameters in the control group changed from basal levels of 14.6 ± 3.4% to 12.4 ± 2.7% and 9.8 ± 1.6% to 12.6 ± 1.6%, respectively. Positive or suspicious cytologic examinations were present in 3/12 (25%) treated cases prior to 4-HPR administration and all subsequently reverted to normal. The same parameter in the control group increased from 4/17 (24%) to 6/17 (35%) during follow-up. Impaired adaptation to darkness was recorded in 4 patients, and transient dermatologic alterations were observed in one-third of the patients, requiring dose reduction in one case. Our preliminary data suggest that 4-HPR may affect the DNA content and abnormal cytology in patients with previously resected superficial bladder cancer.

Key words: 4-HPR, bladder cancer, chemoprevention, DNA flow cytometry, intermediate endpoints, retinoids

Bladder cancer is the fifth most common cancer in the United States, with 49,000 new cases estimated in 1990 [1]. Among newly diagnosed cases, 75–80% of patients initially present with superficial bladder cancer (SBC), which is comprised of three stages: Ta (*in situ* papillary carcinoma), T1 (tumor invading the lamina propria) and TIS (or flat *in situ* carcinoma) [2]. After transurethral resection (TUR) of papillary tumor(s), approximately 40–80% recur, while the incidence of progression to muscle-invasive cancer ranges from 15% to 20%. Importantly, tumors may recur anywhere in the urothelial lining and multicentricity in time and space is observed (polychronotropism, field effect). Although the management of SBC is still controversial, current data indicate that intravesical Bacillus Calmette-Guerin (BCG) is the standard therapy for *in situ* carcinoma and is the most effective prophylactic agent following TUR for recurrent or less differentiated papillary stage pTa and pT1 tumors [2,3]. Administration of BCG is accompanied by a high incidence of side effects, 1–2% of which are severe. Instillation of cytotoxic agents (mitomycin-C, adriamycin, thiotepa, *etc.*) has also been shown to be more

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effective than surgery alone in delaying and reducing the risk of superficial recurrence, although the benefits in terms of disease control are less significant [2]. However, the high recurrence rate and the tendency for multifocality observed in all clinical trials indicate the need for additional treatment.

In recent years, the efficacy of natural and synthetic analogs of vitamin A (retinoids) as inhibitors of bladder carcinogenesis in rodents has been well documented [4–6]. Particularly notable among these studies was the activity of *N*-(4-hydroxyphenyl)retinamide (fenretinide, 4-HPR), which was among the most active and the least toxic of several retinoids tested in the mouse [6]. Pharmacokinetic studies in rodents have also shown that this compound can reach high concentrations in the bladder [7]. In the clinical setting, the activity of retinoids in the prophylaxis of SBC (*e.g.*, etretinate) has already been investigated, but toxicity was often severe and precluded the use of optimal doses [8–10]. Nevertheless, the results of two studies showed that low dose etretinate (25 mg daily) was moderately effective in the prophylaxis of SBC [8,9]. 4-HPR is currently under investigation in Italy in three large, controlled clinical trials for the prevention of breast, skin and oral cancer [11, 12]. Toxicity is limited even after long-term (up to 5 years) intake [13] and is substantially less than other retinoids [14]. This issue is crucial in chemoprevention studies which require chronic administration, particularly in elderly populations afflicted with SBC. For these reasons, 4-HPR appears to be the retinoid of choice for chemoprevention studies in SBC [15].

Much emphasis has recently been given to the search for biomarkers potentially associated with specific stages of the carcinogenic process (intermediate endpoints) in order to overcome the inherent problems associated with using cancer incidence and mortality as study endpoints [16,17]. Two important components in the process of carcinogenesis are genetic instability coupled with increased cell proliferation. Assessment of DNA ploidy and proliferation by flow cytometry (FCM) [18,19], therefore, may be a suitable marker for interim chemopreventive trials. The role of DNA-FCM as a clinical tool has been particularly emphasized in SBC as a diagnostic procedure by using serial bladder washings to non-invasively obtain cell samplings which may represent the entire bladder epithelium [20]. Since retinoids are required to maintain normal proliferation and differentiation of epithelial cells and are able to prevent or revert the development of malignant clones in experimental systems [21,22], we tested the ability of 4-HPR to modulate the DNA content in cells from patients with resected SBC.

MATERIAL AND METHODS

Patients and Study Protocol

The study was initially conceived as a pilot trial to (1) test the feasibility of monitoring the effect of 4-HPR by DNA-FCM and cytologic examination of serial bladder washings and (2) generate data on the toxicity of 4-HPR in a population of elderly patients. Eligibility criteria for study entry were: histological diagnosis of new or recurrent transitional cell carcinoma (stage Ta, T1, TIS—any grade) within the previous 3 years, and no cystoscopic evidence of disease at the time of admission; termination of intravesical treatment at least 2 months before starting administration of the retinoid; age <80 years old, unless performance status = 0 (WHO criteria); absence of general, dermatologic and ophthalmologic contraindications to the use of retinoids; absence of severe metabolic, liver and renal alterations; and written consent from the patient. The study was approved by the Institutional Review Board of the National Institute for Cancer Research, Genoa, Italy. Patients were accrued between October 1989 and July 1991. The main characteristics of study patients are summarized in Table I. The median interval from TUR to 4-HPR administration was 5.5 months (range 0–36) and the median treatment time was 13 months (range 3–31). Previous intravesical treatment consisted of either BCG (Pasteur strain, 150 mg weekly) for 6 weeks or mitomycin-C (40 mg weekly) for 8 weeks. Study treatment consisted of 4-HPR (RW Johnson Pharmacology Institute, Springhouse, PA), at the daily oral dose of 200 mg (two capsules at dinner) with a 3-day drug holiday at the end of each month. The median interval between the end of intravesical treatment and the time of the study entry (first DNA measurement) was 12 months (range 3–36) in the experimental group and 0 months

TABLE I. Patient Characteristics

	4-HPR (n = 12)	Controls (n = 17)
Median age (range)	68 (29–84)	64 (50–82)
Sex (M/F)	11/1	16/1
Stage[1]		
TaG1	–	2
T1G1	3	3
T1G2	5	7
T1G3	4	5
Previous history of intravesicular treatment		
BCG (Pasteur strain)	5	6
Mitomycin-C	4	5
None	3	6
Median follow-up time: months (range)	12 (3–31)	9 (2–22)

[1] Highest stage if recurrent

(range 0–36) in the control group. Adaptation to darkness was checked in all patients at each visit by means of an ophthalmologic questionnaire. In addition, eight patients were tested for dark-adaptation according to the method of Goldmann-Weekers [23], which comprises the assessment over 30 minutes of the minimal light threshold reported by dark-adapted patients after a 5-minute exposure to high luminance (2,100 apostilb) and by electroretinogram (ERG) under photopic and scotopic adaptation, as previously described [24].

At approximately four-month intervals, patients underwent cystoscopy followed by a bladder washing procedure of double irrigation with 5–6 vigorous pulses of 100 ml normal saline through a urethral catheter or a resectoscope sheath. The fluid specimen was immediately mixed and divided for cytologic and FCM analysis. Inasmuch as the study endpoint (DNA content distribution) represents a new model in designing clinical trials and no information on the time course of these variables in a population with SBC are available from the literature, we have observed a control group of 17 age-matched non-randomized patients (7 of which were among those who subsequently received 4-HPR). However, in light of the potential source of bias generated by such a comparison and the different observation time (from first to last washing) between the two groups (different median follow-up time), no formal test of statistical significance has been applied. Moreover, since individual patients had different numbers of washings and none showed more than one variation in the test parameters during the follow-up period (*e.g.*, patients becoming diploid never reverted to the initial aneuploid status or *vice versa*), comparisons were only made between the first and last samples. All values are given as mean ±SE.

Cytology

Bladder washing specimens were centrifuged at 1700 rpm for 10 minutes, the sediment was smeared on two glass slides, fixed in Delauney solution (ethanol-acetone) and stained by Papanicolaou technique. According to the criteria proposed by Koss and Murphy [25,26], four cytological classes were identified (blinded as to treatment): (1) normal transitional cells, includ-

ing reactive cells; (2) atypical hyperplasia, including grade I papillary tumors (WHO classification); (3) transitional cells with suspicious (but not definitively malignant) atypia (severe dysplasia); and (4) intermediate to high grade malignant tumors, including grade II and III papillary tumors and TIS. In reporting the results, classes 1 and 2 were pooled and classified as negative, while classes 3 and 4 were classified as positive.

Flow Cytometry

Staining and DNA measurements. Samples were processed either fresh (within one day of storage at 4°C) or after freezing at –80°C at a concentration of approximately 10^7 cells/ml [27]. Staining was performed using a slight modification of the method of Thornthwaite *et al.* [28]. Briefly, about 10^6 cells either fresh or thawed were centrifuged and then diluted in 1 ml of nuclear isolation-staining solution [NIM-DAPI: 10 mM phosphate buffer in isotonic saline, 1 mM $CaCl_2$, 0.5 mM $MgSO_4 \times 7H_2O$, 1.5% Nonidet P-40 (v/v), 0.2% bovine serum albumin (w/v), and 10 mg/l of 4,6-diamidino-2-phenylindole-2 hydrochloride] (DAPI; Sigma Chemical Co., St. Louis, MO). Trout red blood cells (TRBC) were used as an internal DNA standard; the ratio of the DNA content of TRBC to human DNA diploid G0/G1 cells from the bladder washing specimens was 64.2 ± 2.7%.

The DNA measurements were performed using an arc lamp flow cytometer (model ICP22A; Ortho Diagnostic Systems, Westwood, MA) with suitable filters for DAPI excitation (between 300 and 400 nm) and for DAPI fluorescence emission (between 450 and 490 nm). The fluorescence signals were analyzed using a 1,024 channel PDP 11-23 computer (Digital Equipment Co., Maynard, MA).

Data analysis. Histograms of the number of cells versus DAPI fluorescence intensity (which is proportional to DNA content) were obtained and used to evaluate the DNA content distribution of the bladder washing samples. For each sample a second measurement was performed after addition of TRBC. The coefficient of variation (CV) for the G0/G1 peak was calculated as width at half maximum of the peak divided by the peak mean channel number and the factor 2.35. According to an international convention [29], only the samples with at least two separate G0/G1 peaks were considered aneuploid. The degree of DNA aneuploidy, *i.e.*, the DNA index (DI), was obtained by dividing the mean channel number of the DNA aneuploid G0/G1 peak by the mean channel number of the diploid G0/G1 peak, where the diploid G0/G1 peak was identified in relation to the TRBC peak and was defined as DI = 1.00. In histograms with no apparent aneuploid peaks, the approximate percentage of cells in the G0/G1, S and G2+M phases of the cell cycle was obtained as previously described [30]. Correction of the values for S-phase fraction, G2+M-phase fraction and DNA-aneuploid peak size was performed after visual quantitative assessment of the non-transitional cell component (percentage of non-urothelial cells in 200 cells representative of the whole sample) under the assumption that non-urothelial cells are quiescent, and are therefore diluting only the G0/G1 cell compartment.

RESULTS

The proportion of patients with DNA aneuploid stemlines decreased in the experimental group from 7/12 (58%) to 5/11 (45%) in the course of 4-HPR administration (one patient with an initial diploid profile was no longer evaluable for FCM analysis), while it increased from 7/17 (41%) to 10/17 (59%) in the control group (Fig. 1A). In the 4-HPR group, all patients with initial diploid profile (n = 4) remained stable, while two patients showing initial aneuploidy reverted to normal. In the control group, three patients who were initially diploid became aneuploid, while no patient with initial aneuploidy reverted to diploid during the follow-up period. The behavior of the S-phase and G2+M-phase fractions among patients with stable diploid profiles is illustrated in Figures 1B and 1C, respectively. Mean (±SE) S-phase and G2+M-phase fraction values decreased in the 4-HPR group from 15.2 ± 4.1% to 7.5 ± 3.3% and 10.3 ± 2.2% to 5.2 ± 0.4%, respectively. In the control group the same parameters changed during follow-up from 14.6 ± 3.4% to 12.4 ± 2.7% and 9.8 ± 1.6% to 12.6 ± 1.6%, respectively. Suspicious or positive cytologic examinations were present in 3/12 (25%) cases before 4-HPR treatment and reverted to normal in all cases. The same class in the

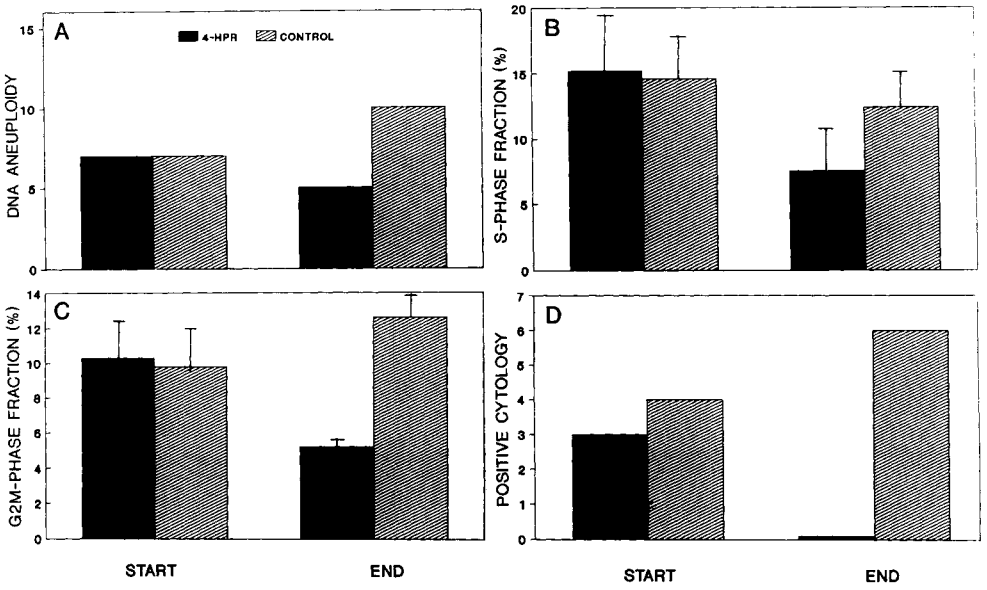

Fig. 1. Variation of flow cytometric [(A), (B) and (C)] and cytological (D) parameters of bladder washings from 4-HPR-treated patients (black bars) and untreated controls (grey bars) from the beginning (basal bladder washing: "START") to the end of the follow-up period (last bladder washing: "END"). On vertical axis: (A) Number of aneuploid cases; (B) Mean % (±SE) of the S-phase fraction of cases which were diploid both at the "START" and at the "END" among the 4-HPR-treated (n = 3) and untreated patients (n = 6); (C) Mean % (±SE) of the G2+M-phase fraction of the same cases as (B); (D) Number of cases with positive or suspicious cytologic examination.

control group increased from 4/17 (24%) to 6/17 (35%) in the follow-up period (Fig. 1D). The time course of FCM-DNA histograms in 4 representative patients receiving 4-HPR is illustrated in Figure 2.

Three patients in the experimental group (25%) complained of decreased adaptation to darkness and four patients (33%) were positive in the dark-adaptation test. Alterations consisted of either a prolonged interval to adaptation (cone phase >8 min) in three cases or a reduced ability of the rods to adapt (increase in the absolute terminal threshold above 4 U log picostilb) in one case. ERG under scotopic adaptation was performed in 8 patients and was abnormal in two (rod phase). Since none of the positive cases were considered to have a severe degree of nyctalopia (taking into account that all four patients were aged >65), treatment was maintained at full dosage in all patients. Dermatologic alterations were observed in 4 patients (33%), consisting of one episode each of mild photodermatosis, skin desquamation, pruritus and skin rash. Treatment was stopped for 3 weeks in the patient with photodermatosis and then reintroduced at half dosage. No alteration in blood tests was recorded in any patient.

DISCUSSION

Interest has increased in chemoprevention as a means of reducing cancer mortality since introduction of the concept by Sporn *et al.* more than 15 years ago [31]. Unfortunately, chemopreventive trials to test the ability of experimental compounds to reduce cancer incidence and mortality require the observation of several thousand patients over a prolonged period. To overcome these limitations, recent interest has focused on developing a more mechanistic approach based on increasing knowledge of the evolving molecular and cellular stages that precede development of invasive cancer [32]. In this context, the identification of biomarkers associated with specific characteristics of carcinogenesis (intermediate endpoints) may allow

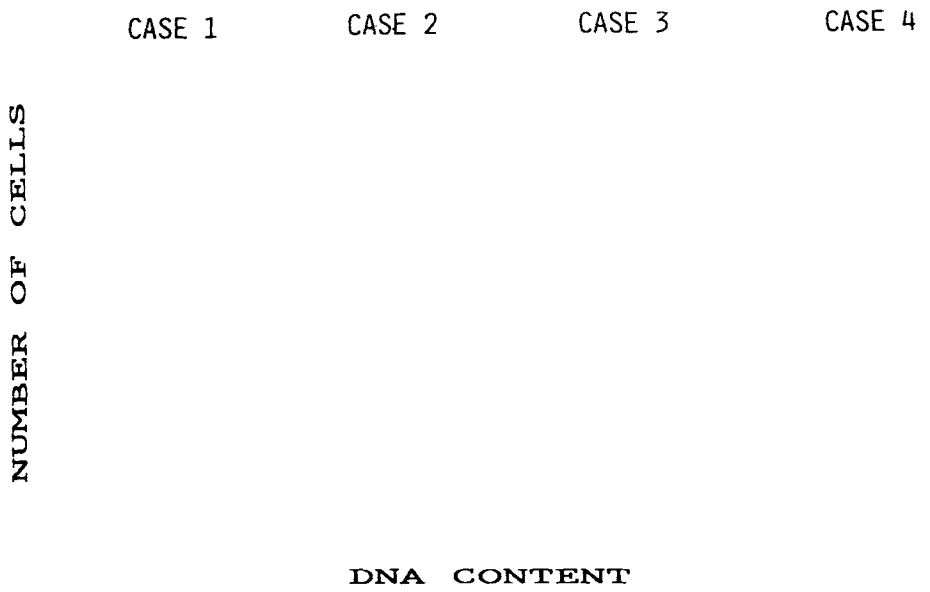

Fig. 2. Examples of DNA histograms of four different patients at the beginning of 4-HPR treatment (top row) and after a time period reported in each panel (bottom row). All histograms represent 10,000 urothelial cells (no inflammatory or squamous cells were found in the washings). Case 1: the aneuploid peak size with DNA Index (DI) = 1.13 decreased from 25% in the basal histogram to 4% after 5 months. Case 2: diploidy was maintained for 10 months and the S-phase fraction decreased from 15% to 2%. Case 3: the aneuploid peak with DI = 0.9 in the basal histogram disappeared and a diploid histogram was maintained for 24 months. Case 4: the basal diploid washing evolved to aneuploidy with a DI = 1.8.

more rapid and less expensive testing of potentially highly active chemopreventive agents [17]. Our first objective was, therefore, to evaluate the feasibility of such an approach in patients with SBC using DNA-FCM to evaluate the activity of 4-HPR, a synthetic retinoid with proven chemopreventive activity in rodent bladder cancer systems [6,33]. Since current data in the literature indicate that DNA-FCM may be a reliable and reproducible indicator of DNA content distribution, our study may provide a model for such new clinical trials in the future.

Our preliminary data suggest that 4-HPR may be able to revert abnormal cytology and modify DNA content distribution both in terms of ploidy and proliferative activity. Previous studies in murine and human breast cancer cell lines have shown that 4-HPR can reduce the S-phase fraction with a concomitant increase in the percent of cells in the G0/G1 phase of the cell cycle [34,35]. Moreover, recent observations indicate that inhibition of neoplastic transformation exerted by 4-HPR and other retinoids (of initiated fibroblasts) may be accomplished by specifically blocking the G0 to G1 transition induced by mitogenic growth-factors (PDGF, EGF) [36], the role of which appears to be crucial in bladder carcinogenic transformation [37]. Control of growth and differentiation pathways may be mediated in part by interactions between specific nuclear receptors, which often induce cellular differentiation and inhibit cell growth, and nuclear protooncogenes, which often stimulate cell growth, *e.g.*, c-*jun*, c-*fos*, c-*myb* [22]. At the molecular level, a major consequence of retinoid administration is induction of the regulatory peptide, Transforming Growth Factor-β, which has a profound inhibitory activity on proliferation and the loss of differentiation in many organs [21].

Side effects were relatively mild despite the advanced age of many patients (median age 68 years) and prolonged retinoid administration (median 13 months), allowing full dose administration in all but one case. Our results confirm the alterations of dark-adaptation previously reported with 4-HPR administration [24,38], which are probably related to the de-

crease in retinol levels [39]. In our case, however, the occurrence of nyctalopia was documented by the Goldmann-Weekers dark-adaptation test and not always observed by ERG, suggesting the higher sensitivity of the former test. In fact, objective alterations of ERG have been reported with experimental doses of 4-HPR 1.5- to 4-fold higher than those employed in our study [24, 38]. Dermatologic alterations were always transient and of grade I, with the exception of one patient in whom a mild form of photodermatosis was observed. In comparison to the toxicity profile observed with 13-*cis*-retinoic acid in elderly patients [14,40], 4-HPR appears much more easily tolerated. The question of its activity in the prophylaxis of SBC remains to be seen in a large randomized clinical trial.

A randomized, controlled clinical trial will soon be initiated by our group to test the efficacy of 4-HPR in modulating the FCM-DNA content. The study is supported by the Chemoprevention Branch, DCPC, NCI. Patients will be randomized and receive either 4-HPR for 2 years at a daily oral dose of 200 mg or no treatment with all patients monitored for an additional third year. Approximately 90 patients (45 per regimen) will be necessary over the 2-year period in order to have an 80% chance of detecting a significant difference (α error = 0.05) if 4-HPR is assumed to be associated with a 30% absolute increase (from 20–30% to 50–60%) in the proportion of "normal" DNA profiles. The definition of normal excludes the presence of aneuploid peaks (including G2+M peaks of >15%, *i.e.*, tetraploid peaks) and the presence of S-phase fraction >12% in the diploid profiles. Cystoscopy and bladder washing will be performed at 3-month intervals for cytologic examination and measurement of FCM-DNA (blinded as to treatment and patient identification). The main endpoint is the proportion of normal DNA profiles at 12 months. The study is not expected to detect significant differences in the incidence of recurrent superficial tumors unless dramatic effects are seen with the retinoid administration (relative risk <0.25).

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