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Local Control of T2/3 Transitional Cell Carcinoma of Bladder Is Correlated to Differences in DNA Supercoiling: Evidence for Two Discrete Tumor Populations[1]

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ABSTRACT

Single cell tumor suspensions were prepared from biopsy and urine samples from 48 patients with muscle invasive transitional cell carcinoma of the bladder. Prior to therapy, samples were irradiated *in vitro* with the condensation of DNA supercoils measured by the light scattered within a flow cytometer. Six months after completing a course of radiotherapy, the *in vitro* data were correlated with the presence or absence of local disease. After 12-Gy irradiation, nucleoid extraction and staining with 50 μg/ml ethidium bromide, 2 predominant forms of supercoiling behavior were seen. Nucleoids scattered either approximately 10% (Type I) or 35% (Type II) more light than unirradiated controls. Those patients with residual disease showed more Type I behavior (21 of 25; 84%) than those patients clear of disease (9 of 23; 39%) ($P = 0.02$). It is proposed that the ability of these tumor samples to adopt positive supercoiling after irradiation is related to a stronger association between individual DNA loops and their attachment to the nuclear matrix. This difference in nucleoid response within these tumor samples may be related both to intrinsic cellular radiosensitivity and, subsequently, to clinical radiocurability.

INTRODUCTION

Within most centers in the United Kingdom, the treatment of muscle invasive bladder cancer commonly involves radiotherapy, with cystectomy reserved for those patients in whom local disease control has not been achieved (1). The latter option is only of benefit for those patients who are fit for surgery with no evidence of distant disease. Using this treatment rationale, 40% 5-year survival rates have been reported, though certain tumors may progress to an inoperable stage while the response to radiotherapy is being assessed (1–3). In a previous study, the incidence of squamous metaplasia and β-human chorionic gonadotrophin content, the former being a useful marker of disease aggressiveness (4), were correlated with local control failure. Used prospectively, such markers would allow the identification of those patients who may not respond to first line radiotherapy and may benefit from receiving either chemotherapy or a cystectomy. The presence of aneuploid tumor populations or variations in S-phase are equivocal predictors of survival (4–6).

Clinically, the most informative indicator of disease-free survival is the gross elimination of detectable tumor 3–6 months after treatment; such patients have a 54–58% 5-year survival rate compared to 17–18% for those with residual disease (1, 4). For these latter patients, however, the time taken to demonstrate a failure in local control permits further growth and dissemination of the tumor. It is possible that those patients with residual local disease after treatment represent a subset of patients with particularly radioresistant tumor. We have attempted to define an additional prognostic marker, available prior to treatment, that is linked to the radiation resistance of individual tumor cells. This test exploits the correlation between radiosensitivity and the ability of DNA to maintain its supercoiled state.

With this technique, cells treated with a hypertonic salt buffer release DNA with its higher order supercoiled structure intact (7). Such residual nuclei, or nucleoids, when stained with a concentration of ethidium bromide greater than 10 μg/ml, contract in size as the ethidium bromide introduces positive supercoiling into individual DNA loops (8, 9). Loops that have been damaged, for example by radiation, fail to contract and the resulting nucleoids are therefore larger (10). These variations in size can be monitored by centrifugation, microscopy, or in our case flow cytometry (10–12). Using the last technique, the amount of laser light scattered from individual nucleoids is measured. We and others have shown that relatively radiosensitive cells, of rodent or human origin, show a reduced ability to maintain a compact positively supercoiled structure after irradiation (11, 13–16).

We now report the conclusion of a clinical study in patients undergoing radical radiotherapy for muscle-invasive transitional cell carcinoma of the bladder. A preliminary report has appeared elsewhere (17). In this study, a tumor biopsy was taken from each patient prior to treatment and irradiated *in vitro*, and the light scatter from discrete nucleoids analyzed by flow cytometry. These data were then correlated with local tumor control, 6 months after completion of therapy.

MATERIALS AND METHODS

Patients. Only patients with histologically proven, muscle invasive (T2 or T3), transitional cell carcinoma of the bladder were recruited into this study. All patients underwent visually complete transurethral resection of their tumor prior to radiotherapy. Tumor samples for analysis were received either at surgery or from voided urine immediately prior to resection. Patients were subsequently treated with a radical course of external beam fractionated radiotherapy, of approximately 52.5 Gy in 20 fractions. Six months after completion of radiotherapy, each patient underwent cystoscopic examination to determine the presence or absence of persistent disease.

Sample Collection and Preparation. Transurethral samples of tumor were transported in saline to the laboratory within 3 h of excision. Each sample was sliced into approximately 2-mm cubes with crossed scalpel blades and incubated in 4 ml collagenase (2000 units/ml in Ham's F-10 nutrient medium; both from Sigma, United Kingdom) at 37°C for 1 h. The specimen was then passed through 38-μm mesh muslin gauze, washed twice in medium, and resuspended in Ham's F-10 supplemented with 10% fetal bovine serum to give a suspension of single cells. Samples of tumor cells received from voided urine were centrifuged (800 × *g* for 5 min), washed twice in the above media, and resuspended to give a suspension of single cells. An aliquot was taken from all samples, mounted on microscope slides, and separately stained with both Giemsa and hematoxylin/eosin. The proportion of tumor cells present was allocated by inspection into either >50% or <50% of the total nucleated population. Some samples were fixed with a 0.07% solution of paraformaldehyde in saline and stained with 20 μg/ml propidium iodide, and a histogram of DNA content accumulated from analysis of 10,000 cells.

Nucleoid Analysis. A Becton Dickinson FACS 440 jet-in-air flow cytometer was used as described previously (12). All samples were run at standard settings of sheath fluid pressure (15 p.s.i.), photomultiplier gain, and voltage after preliminary optical alignment using 11- and 15-μm polystyrene beads.

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The standard operating conditions were chosen to place the forward scatter data of both control and irradiated nucleoids within the 256-channel dynamic range of the photomultiplier detectors. To generate nucleoid scatter data, the position of the forward obscurator bar was found to be critical. This bar is situated in front of the forward light scatter detector and is used to block the undeflected laser beam. To collect low angle light scatter, this bar is adjusted to present the minimum area to the incident laser beam, consistent with blocking direct light access to the forward scatter detector.

The cells to be used were divided into 1-ml aliquots at approximately 1×10^6 cells/ml and placed on ice. Samples were then irradiated at 0°C with 3–18 Gy from a ^{60}Co-γ source and replaced on ice. Immediately prior to analysis, and within 45 min of irradiation, 100 μl (1×10^5) of cells were mixed with 1 ml of lysis buffer containing 2 M NaCl, 10 mM disodium EDTA, 10 mM Tris buffer, and 0.1% Triton X-100. Quadruplicate samples from irradiated and control cells were stained with either 10 or 50 μg/ml ethidium bromide immediately before analysis. Data from 10,000 nucleoids were accumulated for each sample, triggering data acquisition on red (DNA) fluorescence. Forward scatter, side scatter, and red fluorescence histograms were recorded.

RESULTS

Eighty-eight patients presented with T2/3 transitional cell carcinoma of bladder during the time of this study. In addition to tumor cells, most single cell preparations contained RBC and polymorphonuclear neutrophils. Those samples in which no tumor cells were positively identified by independent pathological criteria (20 of 88; 19 of 20 from urine) were excluded from analysis, as were those samples from patients with incomplete follow-up, or who did not complete treatment (Table 1). The number of samples without apparent tumor is probably artificially high due to the difficulty of identifying isolated aberrant cells without guidance from surrounding gross histology. Most samples (36 of 48) were obtained from urine, and 12 by biopsy. Nine of the patients from whom biopsies were taken and 16 of those from urine samples had persistent disease when examined 6 months after completion of treatment. The patient characteristics are shown in Table 2.

Thirty-two of the samples were also separately exposed to both 10 and 50 μg/ml of ethidium bromide. The lower dose produces extension of individual supercoiled loops, resulting in nucleoids of a larger diameter. By reference to the maximum relaxation/contraction seen in other systems after ethidium bromide titration, staining with 10 μg/ml of ethidium bromide approximates the maximum DNA loop size (20). In each case, the radiation-induced changes were less than this maximum; staining with 10 μg/ml compared to 50 μg/ml of ethidium bromide produced an increase in light scatter of +93.5% (range, 14.4 to 350.5%), whereas after a 12-Gy irradiation and staining with 50 μg/ml of ethidium bromide, the mean increase was +16.5% (range, -1 to +68%). We have previously shown that repetitive analysis of the same sample can generate a S.E. of less than 5%; a similar precision is found within quadruplicate sampling here (17). The reason for the wide range in light scatter after ethidium bromide staining alone is not known, though biological variation, particularly the amount of DNA available to undergo changes in supercoiling, may account for some of the differences. This possibility is supported by the correlation between the amount of light scatter induced by ethidium bromide and radiation in both patient groups (Fig. 1). No significant correlation was found between maximum loop relaxation (as shown by staining with low dose ethidium bromide) and clinical outcome ($P = 0.1$, t test) (Fig. 1).

Table 1 *Criteria for patient exclusion*

Reason for exclusion	Patients (%)
No pathologically identifiable tumor in sample	20 (23)
Palliative therapy only	6 (10)
Did not complete treatment	7 (8)
Lost to follow-up	7 (8)

Table 2 *Patient characteristics as determined by the presence or absence of disease after treatment*

Parameter	Residual tumor	Clear of tumor
Median age (range)	74 (45–85)	75 (41–88)
Sex	20 M, 5 F	23 M

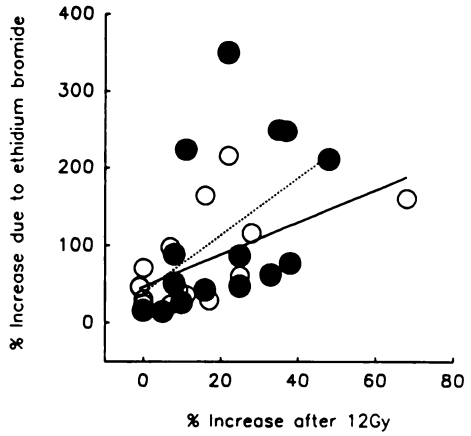

Fig. 1. Percent increase in nucleoid light scatter after irradiation of cells with 12 Gy, compared to that induced in unirradiated nucleoids using 10 μg/ml ethidium bromide to unwind individual supercoiled loops. In each case, data are referenced to unirradiated control nucleoids stained with 50 μg/ml. The data are split into those patients who either did (●) or did not (○) achieve local tumor control, 6 months after treatment. *Curves* plotted are first order regression lines, and each *slope* is significantly different from zero. —, tumor control ($P = 0.01$); · · · ·, tumor persistence ($P = 0.04$). There was no significant correlation between patient outcome and the ability of low dose ethidium bromide to unwind DNA supercoils ($P = 0.1$).

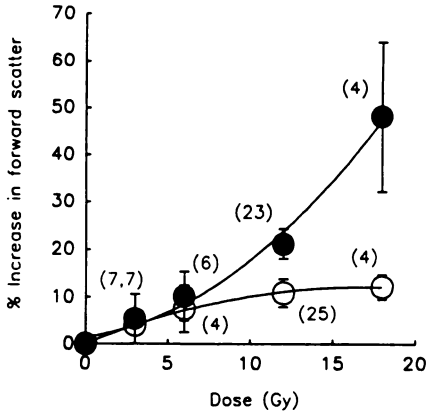

Fig. 2. Dose response of nucleoid light scatter from individual patient tumor cell samples. *Points* were analyzed in quadruplicate, and the means used to fit a second order regression equation. ●, patients whose tumor was controlled 6 months after treatment; ○, patients with persistent disease. *Bars*, SE; *numbers* in *parentheses*, sum of patients at each *point*.

Thirty-one samples were analyzed by flow cytometry for differences in ploidy status, determined by inspection of the flow histograms. In those patients clear of disease 6 months post-therapy, 5 of 13 (38%) were aneuploid, whereas 10 of 18 (58%) were aneuploid in those patients with residual disease. The presence of an aneuploid population was not significantly related to the clinical response as demonstrated by the χ^2 statistic.

The complete nucleoid dose response over the 3–18-Gy range is shown for patients who either did or did not have persistent disease (Fig. 2). At both 12 and 18 Gy, those nucleoids taken from patients who were clear of tumor, 6 months after completing therapy, exhibited enhanced light scatter compared to those samples taken from patients who failed to achieve local control ($P = 0.02$ at 12 Gy). We attribute

this increase in light scatter after irradiation to the inability of looped DNA to maintain DNA supercoiling after ethidium bromide intercalation. The data taken from tumor cells irradiated with 12 Gy were also analyzed as a frequency distribution (Fig. 3). Patients who failed to achieve local control were associated with nucleoids that were approximately 10% larger after irradiation and ethidium bromide staining, called here a Type I response. In contrast, patients whose tumor was controlled showed a biphasic pattern, with an additional larger nucleoid response of approximately 35%. This is called a Type II scatter response.

As described above, the samples collected contained a variable amount of nucleated cells of non-tumor origin. The criteria used to record tumor cells were rigorous, and substantial numbers of samples were rejected as having no tumor content, in itself an unlikely event. We would expect that any error in detecting tumor would tend to underestimate the amount present. To determine the effect of the contaminating normal material, the nucleoid scatter of samples was compared to tumor content. Those samples with the most positively identified tumor reflected the strongest correlation between nucleoid behavior and local tumor control, supporting the interpretation made on the entire data set (Fig. 4). It is clear that for future studies, a rigorous attempt at excluding non-tumor cells must be made.

DISCUSSION

The amount of light scattered by nucleoids is approximately proportional to their size, and this is a good relative indicator of changes in nuclear supercoiling (12, 18). To confirm the nature of the DNA organization detected by this assay, 32 of the 48 samples were exposed to both high (50 μg/ml) and low (10 μg/ml) concentrations of ethidium bromide. All samples expressed an increased mean forward scatter at the lower concentration, consistent with the unwinding of individual DNA loops presenting a larger target for laser light scatter (Fig. 1) (9). The presence of DNA strand breaks also results in larger nucleoids, as such affected loops cannot maintain the tension induced by either their inherent supercoiling or high doses of ethidium bromide (10). In this study, those patients who achieved local control were associated with a larger increase in nucleoid light scatter after sample irradiation than those whose tumor was still detectable 6 months after completing treatment ($P = 0.02$ at 12 Gy; Figs. 1 and 2). It is unlikley that these differences are due to the presence of different numbers of strand breaks produced by the same dose, as their effect on light scatter differs 40-fold between samples (Fig. 1). There is also evidence for 2 separate populations of tumor cell responses within the patient group studied, at least as seen after 12-Gy irradiation (Fig. 2). We suggest,

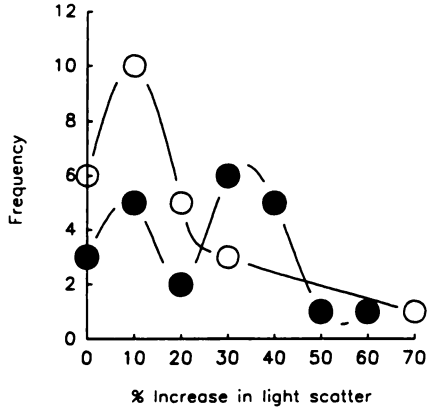

Fig. 3. Frequency response of nucleoid light scatter after 12 Gy divided into decile percentage increments. ●, patients controlled by the radiation; ○, patients with residual disease.

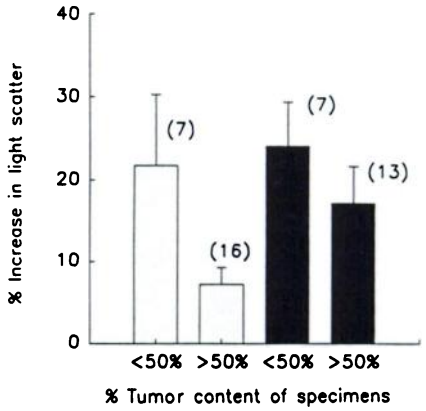

Fig. 4. Percentage increase in nucleoid light scatter produced by 12-Gy irradiation of intact cells, correlated to those patients with either > or < 50% tumor within the *in vitro* irradiated specimen. ■, patients clear of disease after treatment; □, patients with residual disease. *Bars*, SE; *numbers* in *parentheses*, number of patients in each group.

with reference to experimental data from cell culture systems discussed below, that the enhanced local control and nucleoid behavior are both related to alterations in intrinsic cellular radiosensitivity.

After irradiation of rodent V79 cells, ethidium bromide-stained nucleoids derived from the relatively radiosensitive monolayer growth form are both larger and more fragile than those isolated from cells grown as radioresistant spheroids (16). In this system, neither the induction or repair of single and double strand breaks, nor stage of the cell cycle, can explain the differences in radiosensitivity or nucleoid response (19). A similar correlation between radiosensitivity and the ability to maintain positive supercoiling has been reported within human squamous cell carcinoma cell lines, ataxia telangiectasia fibroblasts, and variants of the LY5178 murine leukemia line (11, 14, 15). All of these data support a link between the ability of extracted DNA to maintain positive supercoiling and relative radiosensitivity. In this study, such a hypothesis would indicate that transitional cell carcinoma of bladder may be divided into at least 2 subtypes, according to cellular radiosensitivity. Those samples able to maintain positive DNA supercoiling after cellular irradiation and nucleoid extraction appear to represent a radioresistant phenotype associated with the failure to locally control this disease. If this is correct, it would provide at least one reason why some patients treated with full course radiation possess residual tumor, due to inherent radioresistance.

The differences in nucleoid response between each patient group, and in the cell lines reviewed above, may reflect a different organization of DNA loops within relatively radioresistant and radiosensitive cells. Two possible mechanisms are proposed that may explain the physical data presented here. An increase in DNA loop size would allow a larger total DNA relaxation, as more DNA would be affected per break (13). However, no such radiosensitivity-linked alteration in DNA loop size has been reported within either human squamous carcinoma cell lines or ataxia telangiectasia fibroblasts (14, 20). Also in this study, there was no correlation between maximum loop size, as shown by the difference in response between nucleoids stained with either 10 or 50 μg/ml ethidium bromide, and clinical outcome (Fig. 1). There is, however, a general correlation between the ability of either radiation or low dose ethidium bromide to induce relaxation, and this may represent the different capacities of individual samples to undergo supercoiling changes of any type. As an alternative mechanism, the loops may be of similar size, but the strength of their attachment to the core protein matrix may differ (21). In this case, those nucleoids with the weakest attachment may show an enhanced relaxation after radiation exposure by the production of multiple relaxed loops *in vitro*, due to the propagation of supercoil unwinding through the sites of DNA attachment to the matrix. This interpretation best fits the data

gained using *in vitro* cell culture systems where loop relaxation may be followed through time until a plateau is reached, consistent with increasing numbers of DNA loops unwinding in response to a priming radiation lesion (20).

By definition, the propagation of DNA loop relaxation would proceed through the sites of DNA attachment at the nuclear matrix, the same sites where DNA synthesis is initiated (22, 23). Matrix attachment regions contain a stable DNA configuration where the bases are unpaired over at least a 200-base pair region (24, 25). Adoption of this DNA configuration occurs in AT-rich, sequence-specific regions of DNA that include both homologies with the topoisomerase II consensus sequence and are associated with strong binding of DNA to the nuclear matrix. Experimentally increasing supercoil tension *in vitro* using ethidium bromide can alter the length of this region, and such changes, if they occur *in vivo*, may modulate the affinity of this region for the nuclear matrix (24). Endogenous changes of this type may be responsible for the differences in DNA-matrix affinity proposed here. While it is possible to start to analyze the potential physical constraints to supercoiling, the biological consequences of any such changes *in vivo* are less clear. Cramp *et al.* (26) have studied the association of newly synthesized DNA with its template strand. In their study, newly synthesized DNA from radiosensitive cells was preferentially single stranded when compared to radioresistant cell lines. This suggests a physically weaker association of DNA with its template within radiosensitive cells at the matrix site linked to DNA synthesis initiation. Also, the majority of cell lines studied that have demonstrated a link between radiosensitivity and nuclear supercoiling are also defective in the repair of DNA double strand breaks (11, 27–29). It is not yet clear if, or how, deficient DNA double strand break repair is linked to alterations in nuclear supercoiling behavior through either physical and/or biochemical differences expressed at the matrix attachment region (30).

In its present form, the clinical utility of this technique is limited by its lack of specificity. At least one confounding factor is the contamination of tumor samples with normal epithelial cells (Fig. 4). For at least some tumors, restricting analysis to aneuploid cell populations would exclude such normal cell contamination. This would include 48% of patients in whom this factor was measured in this study, though others have reported between 37 and 94% aneuploidy within this tumor type (5, 6). Such tumor specificity may be obtained for those samples containing aberrant amounts of DNA by the coanalysis of a normal population, such as lymphocytes, or normal tissue adjacent to the biopsy site. Differences in ethidium bromide fluorescence of nucleoid DNA derived from aneuploid tumor cells may then be used to electronically reject those data with a normal DNA content.

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