Comparison of Tissue Disaggregation Techniques of Transitional Cell Bladder Carcinomas for Flow Cytometry and Chromosomal Analysis


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DNA index (DI) measurements and chromosomal analysis of 42 transitional cell carcinomas were done after mechanical and enzymatical disaggregation of the tumor specimens. The results obtained with these different disaggregation techniques were compared in the 33 cases (79%) that showed recognizable chromosomes. The enzymatically obtained cell suspensions could not be used for chromosomal analysis after short-term culture of 24 hours. In four cases, the DI after enzymatic treatment could not be estimated. In most cases, the DI obtained from the tumor cells was similar for both aggregation techniques, with the exception of four cases of enzymatically treated cell suspensions in which the DI could not be estimated. The average DI of the aneuploid tumors was 13% higher than the corresponding chromosome count.

In 19% of the aneuploid tumors the proportion of aneuploid cells could not be measured after enzymatical treatment. In the remaining suspensions the proportion of diploid cells was higher after enzymatical disaggregation than after mechanical treatment. It is concluded that for flow cytometric and direct chromosomal analysis of bladder tumors, the mechanical disaggregation technique is most suitable.

Key terms: Tissue disaggregation, bladder carcinoma

Flow cytometry and chromosomal analysis have been described as important methods for the examination of bladder carcinomas (10–12,23). The degree of ploidy and the proliferative activity of the tumor can be used as additional parameters in patient treatment (3,20). The same significance may be adjudged to the detection of chromosomal abnormalities in tumor specimens (6,15,17,18). Essential for proper flow cytometric (FCM) investigations and chromosomal analyses are optimal single-cell suspensions of the (malignant) tissues under examination. Current methods for disaggregation of tumor specimens include mechanical and enzymatical treatment of the tissue. It has been reported that the proportions of aneuploid cells in cell suspensions obtained by mechanical disaggregation were higher than in those obtained after enzymatical disaggregation (5,8). Differences may also be due to different enzymatical methods (4).

In this study the DNA content of tumor cells in the G0/G1 phase after mechanical and enzymatical disaggregation was measured. These data were correlated with histological as well as cytogenetical data of the same transitional cell carcinomas. The percentages of diploid cells in the G0/G1 phase present in the cell suspensions after the two disaggregation techniques were compared. The effects of disaggregation procedures and the usefulness of the different cell suspensions for FCM and chromosomal analysis are discussed.

MATERIALS AND METHODS

Forty-two tumor specimens, 38 primary and 4 recurrent ones, from 31 male and 7 female successive patients with carcinoma of the urinary bladder were investigated. They could be divided into tumor stages as follows: 14 pT2, 11 pT1, 17 pT2–pT4 tumors. The tissues were collected after transurethral resections.

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One part of each biopsy was used for pathologic examination, while the remaining part was used for chromosomal and flow cytometric studies. Clinical staging of the tumor was done according to the rules of the Union International Contre le Cancer (13), and grading was done according to the WHO system (24). Staging and grading were as follows: \( \gamma \)T1 tumors are noninvasive; \( \gamma \)T2 tumors show no invasion beyond the lamina propria; \( \gamma \)T2–\( \gamma \)T4 tumors show invasion from superficial muscles to neighbouring organs. Grade 1 tumors show hyperplasia but are highly differentiated. Grade 2 tumors show moderate polymorphism, nuclear atypia, and hyperplasia of the epithelium. Grade 3 tumors show severe abnormalities, sometimes to the extent that the tissue is no longer recognizable as urothelial tissue.

**Disaggregation Procedures**

The specimens were collected and transported in the following medium: 10 ml RPMI 1640 plus 17% fetal calf serum (FCS), 50 \( \mu g \) gentamicin/ml, 100 U penicillin/ml, and 100 \( \mu g \) streptomycin/ml.

For mechanical disaggregation, the tissue was minced by scraping and cutting in a petri-dish and was filtered through a 100-\( \mu m \) nylon filter (Ortho Diagnostic Systems, Beersse, Belgium). The cell suspensions were divided in two parts, one for short-term or long-term chromosomal analysis and the other one for flow cytometry. For the latter purpose, the cells were centrifuged at 400g for 7 minutes. Then, 70% ethanol (\(-20^\circ C\)) was added rapidly to the cell pellet under constant shaking. The final concentration was about 3 × 10^5 cells/ml ethanol. At this stage, the fixed cells could be stored several weeks at \(-20^\circ C\).

For mechanical disaggregation, the specimen was cut into small fragments and incubated 1–2 h at 37\(^{\circ}\)C in the medium with 0.8 g/100 ml collagenase II (Worthington, Freehold, NJ). The cell pellet was washed with medium, filtered, and centrifuged. This washing step was repeated three times, and the cell suspension was divided into two parts, one for short-term or long-term chromosome culture and the other one for flow cytometry. For the latter purpose, the cells were fixed in 70% ethanol at \(-20^\circ C\), as mentioned above.

For direct chromosomal analysis, the specimens were collected and transported in 10 ml sodium citrate + 0.5 \( \mu g \) colcemid/ml and disaggregated mechanically.

**Chromosomal Analysis**

For chromosomal analysis one or more of the following techniques were applied:

- **Direct method (in preparation).** Briefly, the tissue specimens were collected and disaggregated as mentioned above. After incubation in Hanks BSS again, a colcemid solution was added (2 \( \mu g \)/ml Hanks BSS). Hypotonic treatment was done with a mixture of 5 ml FCS and 24 ml 0.052 M KCl. Fixation was performed with acetic acid–methanol 3:1.

- **Short-term culture (19).** Briefly, the cells obtained after the disaggregation procedures as described were incubated for 24 hr in the medium. Metaphase arrest with colcemid 0.1 \( \mu g \)/ml was followed by hypotonic treatment with 0.075 M KCl and fixation in methanol–acetic acid 3:1.

- **Long-term culture (9).** Briefly, the cells obtained after the disaggregation methods as described were incubated for 1–2 weeks in flasks containing the culture medium. Methotrexate synchronization was done, followed by colcemid arrest, hypotonic treatment, and fixation.

All slides were stained with Giemsa. C-banding and, when enough appropriate metaphases were available, G-banding was performed. Metaphases were photographed and analyzed according to the standard rules. From every tumor, the chromosomal range, the modal number, the distribution according to ploidy (\( n = 23 \pm 1, 2n = 46 \pm 11, 3n = 69 \pm 11, \text{etc.} \)), and the presence of (double) markers and marker chromosome(s) were recorded (see Table 1). In those cases in which metaphases were obtained with the direct method as well as with the short-term culture the results were pooled.

Our detailed chromosomal observations will be published elsewhere (in preparation).

**Flow Cytometry**

For DNA measurements the sample was stained with the fluorochrome propidium iodide (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs (PI), and thereafter the cells were treated with RNAs 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**RESULTS**

**Normal Bladder Tissue (7 Cases)**

The DI after mechanical disaggregation was 1.01 (range 0.95–1.07) with a S.D. = 0.04. After enzymatical disaggregation it was 1.00 (range 0.93–1.09) with a S.D. = 0.04.
Table 1
Flow Cytometric and Chromosome Data of Cell Suspensions Disaggregated by Mechanical and Enzymatical Procedures in Relation to Stage and Grade

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*DI<sup>1</sup>, DNA index after mechanical disaggregation; DI<sup>2</sup>, DNA index after enzymatical disaggregation; mn, modal No.; dm, double minutes M, marker chromosome(s).

The percentage of G0/G1 diploid cells after mechanical disaggregation was 82.5 (range 75.4-91.4) with a S.D. = 3.6. After enzymatical disaggregation it was 86.7 (range 71.1-94.5) with a S.D. = 4.7.

**Bladder Tumors**

Of the 42 tumor specimens, analyzable metaphases were obtained in 33 cases (79%). The metaphases were obtained with the direct method and/or the short-term culture of 24 h in two cases only after a long-term culture.

Enzymatical disaggregation did not result in analyzable metaphases after a 24-h culture, but in two out of five cases the long-term culture was successful. The DI could be obtained in all the specimens after mechanical disaggregation and in 36 cases after enzymatical treatment. The mean coefficient of variation (CV) for the G0/G1 peak was 5.8 (range 3.5-9.3) after mechanical disaggregation and 6.2 (range 2.4-9.4) after enzymatical disaggregation, respectively. As the study was restricted to the 33 cases with recognizable metaphases, the DI of both pT1 and also of two pT2-pT4 tumor suspensions could not be examined after enzymatical treatment of the specimens. In five suspensions, the percentage of diploid cells in the G0/G1 phase could not be estimated after enzymatical disaggregation of the specimens, owing to a large amount of debris.

**pTa Tumors (12 Cases)**

The DI of all the cases was within the normal range (= 2c) after mechanical disaggregation (Table 1). After enzymatical treatment the DI of one case (No. 5) was slightly higher than normal.

The modal chromosome number was diploid in eight cases, including the two in which only long-term cultures were applied, and hypodiploid in four cases. These
12 samples were obtained from ten patients. Two specimens had a wide range in chromosome number (Nos. 5 and 8).

Marker chromosomes of different types could be recognized in six cases. In three out of 12 cases, neither numerical nor structural abnormalities could be observed.

By comparison of the percentages of diploid cells present in the single-cell suspensions after the two disaggregation methods were applied, no significant difference was seen (p = 0.41; Wilcoxon test, paired case). The mean percentage of diploid cells after mechanical disaggregation was 85.9 (S.D. = 4.5), and after enzymatical disaggregation it was 84.8 (S.D. = 3.8).

**pT1 Tumors (8 Cases)**

The DI after mechanical disaggregation was normal in three cases and varied from about 3c to nearly 5c in the other ones. After enzymatical treatment the DI was normal in the same three cases as above. In three other cases the DI varied from about 3c to about 4c. In two cases the DI could not be measured, owing to marked reduction of the fraction of G0/G1 cells. Comparison of the DI's obtained with the two disaggregation methods showed that the results were identical within a 10% deviation, with the restriction that after enzymatical treatment in two cases the DI could not be measured.

The modal chromosome number varied from hypodiploid to near-tetraploid. All the specimens had an abnormal chromosome constitution. Especially in the aneuploid tumors, a wide range was seen, and cells with different levels of ploidy were present. Marker chromosomes of different types were present in six out of eight cases. In one specimen a lot of double minutes could be observed.

The DI in four out of five cases with an abnormal DNA value was higher than the corresponding chromosome counts. This difference varied from 12% (No. 18) to about 23% (No. 20).

Comparison of the percentages of diploid cells present in the single-cell suspension after mechanical (mean 42.1 with a S.D. = 30.3) or enzymatical (mean 65.8 with a S.D. = 21.3) treatment showed that after enzymatical treatment significantly more diploid cells were present in the cell suspension (Fig. 1) (p = .04). From two suspensions these percentages could not be estimated owing to bad quality of the cell suspensions.

**pT2-pT4 Tumors (13 Cases)**

The DI after mechanical disaggregation varied from about 3c to nearly 5c. In one case (No. 26) two different populations were found. After enzymatical treatment the DI could not be estimated in two cases, owing to strong reduction of the G0/G1 cells. In the remaining cases the DI varied from about 3c to 5c. Also in this group of tumors, the DI's obtained with the two disaggregation methods were identical.

The modal chromosome number varied from diploid to about tetraploid. All the specimens showed an abnormal chromosome constitution. It is conspicuous that most
Infiltrating tumors (pT3-pT4) showed a modal number in the triploid range. Marker chromosomes of different types could be observed in ten out of 13 cases. In one specimen many double minutes were seen.

The DI as estimated by flow cytometric analysis was in all cases higher than the corresponding chromosome counts. This difference ranged from 4% (No. 20) to about 24% (No. 30), with an average of 12%. Comparison of the percentages of diploid cells obtained after mechanical treatment (mean 26.9 with a S.D. = 13.0) and after enzymatic treatment (mean 62.7 with a S.D. = 17.2) showed that after enzymatic treatment significantly more diploid cells were present in the suspension (p = .004). In two suspensions these percentages could not be obtained, owing to bad quality of the cell suspensions.

**Cell Morphology**

Four cell suspensions, one from a non-infiltrating tumor and three from deeply infiltrating tumors, obtained after mechanical or enzymatic disaggregation were examined for cell morphology. The percentage of epithelial cells was reduced after enzymatic treatment. The enzymatic treatment also had a degenerative effect on tumor cells.

**DISCUSSION**

Flow cytometric investigations and chromosomal analyses need optimal single-cell suspensions. Bladder tumor tissue is often very adherent, and a single-cell suspension is often difficult to prepare by mechanical disaggregation. Therefore, enzymatic disaggregation is frequently used. In this study, the usefulness of mechanically and enzymatically obtained cell suspensions for flow cytometry and chromosomal analysis were compared.

Flow cytometry gave data in all cases examined after mechanical disaggregation. In four of the 33 cases compared, the DI could not be examined after enzymatic disaggregation. The DI of the G0/G1 phase of the tumor cells after both disaggregation methods was, with a marginal exception, identical.

The DI of the pTa tumors was 2c. In superficially infiltrating tumors (pT1), normal as well as abnormal DNA values were found, whereas in deeply infiltrating tumors only abnormal DNA values were seen. This agreed with others (23).

For chromosomal analysis, analyzable data were obtained in 33 of the 42 cases (79%). In our hands, it appeared that enzymatically obtained cells were not suitable for the 24-hour short-term culture. For long-term culture we agree with Wake et al. (22) who found that, after enzymatical disaggregation, a large number of cell attached to the bottom of the culture flasks. We do not prefer this long-term culture; since it is time consuming, it is uncertain whether all the cells present in the initial cell suspensions are able to grow, and if so, it is uncertain whether they grow with the same speed.

For this reason we remain in doubt about the representativity of the chromosome constitution obtained after long-term culture. Owing to technical improvements at this moment, it is possible to obtain chromosomes in more than 90% of the tumor specimens, with a direct technique, after mechanical disaggregation.

In cell suspensions, epithelial as well as nonepithelial cells are present. For this reason, in order to reduce this risk of false-negative karyotypes, a great number of cells must be analyzed chromosomally. However, when a few chromosomally abnormal cells among a large number of normal ones are found, it may be assumed that these cells represent the malignant part of the tissue, as soon as two or more cells show the same abnormalities.

The mean value of the DNA content of abnormal pT1–pT4 tumor cells, as estimated flow cytometrically, was 13% above the value expected from the corresponding chromosome count, assuming that the average chromosomal DNA is 1/46 of the mean DNA content of the normal diploid genome. In normal bladder tissue, a 12% deviation is accepted (DI = 2c ± 3 S.D.). When this percentage also is used in aneuploid tumors, the DI measured is still above or in the upper range of the DI expected on the basis of the chromosome count. This difference has been described earlier (11,23). Contribution of double-stranded RNA is excluded, since a RNAse treatment was applied before flow cytometric analysis of the cell suspensions. A disproportionate increase of large chromosomes, as present in many tumors, can not explain this difference, as the DI of small chromosomes did not deviate from normal diploid cells (21). Premature chromosome condensation also focused attention on the need to explain this difference (3). However, no integral explanation for the difference between the DNA values and cytogenetic data could be obtained, and therefore this phenomenon needs further investigation.

Another difference between both methods of analysing the cells was noted with respect to the chromosomal range. By means of FCM analysis, tumors with two or more abnormal cell lines were rarely observed. It is possible that cells with an abnormal DNA value were masked by the S phase in flow cytometric examination. Chromosomal analysis, especially of infiltrating tumors, showed a great range in the chromosome counts. Possibly this represents stem line(s) with side line(s); however, without banding analysis, it is impossible to discriminate among such lines. Nevertheless, it remains worthwhile to examine the clinical significance of this wide distribution.

When the percentages of diploid cells obtained with the two disaggregation techniques were compared, it appeared that in the aneuploid tumors a higher percentage of diploid cells was present in the cell suspension after enzymatic treatment. In other words, the percentage of aneuploid cells was higher after mechanical disaggregation than after enzymatic disaggregation. This difference is probably due to an increase in the
release of stromal cells into the cell suspension as a result of collagenase treatment. The possibility can not be excluded that other enzymes would yield other results. Also, many tumor cells were degenerated. Frankfurt et al. (6) found that in 14 of the 16 aneuploid tumors the proportion of aneuploid cells was higher in the suspensions that were obtained mechanically. In the remaining two cases, enzymatically obtained suspensions contained a slightly higher percentage of aneuploid cells. In three of their cases, the aneuploid cells, which were observed in the DNA histogram after mechanical disaggregation, were not evident in the histogram of enzymatically obtained cell suspensions from the same tumors. In our study, in all of the 15 tumors with an abnormal DI that could be compared, the percentage of aneuploid cells after mechanical treatment was higher than after enzymatic disaggregation. Moreover, in four (=19%) cases of infiltrating tumors, the percentage of aneuploid cells could not be obtained after enzymatic treatment.

Small numbers of aneuploid cells in enzymatically obtained cell suspensions are easily masked by the released nonepithelial cells. Also, in multiparameter analyses, for instance in the determination of percentages of S phase and G2M phase, stromal and inflammatory cells disturb these estimations. To avoid this problem, mechanical disaggregation can be applied, although a better procedure for discriminating the epithelial from the nonepithelial cells is to apply cytokeratin antibodies in a two-dimensional FCM analysis (7, 16).

From our results we conclude that disaggregation of bladder tumor tissue with the enzyme collagenase II is not suitable for short-term chromosome culture. For flow cytometric analysis it does not influence the DI, as compared to mechanical disaggregation, but in four cases the DI could not be estimated after enzymatic treatment. The percentage of diploid cells is higher after enzymatic treatment of aneuploid tumor specimens. Mechanical disaggregation must therefore be preferred to enzymatic methods in the preparation of cell suspensions to be used for FCM and chromosomal analysis without previous culture.

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