Cell Size, DNA, and Cytokeratin Analysis of Human Head and Neck Tumors by Flow Cytometry

J.Th. Bijman, D.J.Th. Wagener, J.M.C. Wessels, P. van den Broek, and F.C.S. Ramaekers
Department of Internal Medicine, Division of Medical Oncology (J.Th.B., D.J.Th.W.), Division of Hematology (J.M.C.W.), Department of Otolaryngology (P.v.d.B.), Department of Pathology (F.C.S.R.), St. Radboud University Hospital, 6525 GA Nijmegen, the Netherlands

Received for publication May 21, 1985; accepted August 4, 1985

Cell subsets have been discriminated in cell suspensions derived from 37 human head and neck tumors by means of light scatter, DNA, and cytokeratin flow cytometry (FCM). Cell dispersion was performed overnight at 4°C in two different enzyme mixtures, i.e., trypsin/dithioerythritol and collagenase/DeNase, under slight agitation of sliced tumor tissue. Cells were examined before and after fractionation on a discontinuous low-density bovine serum albumin (BSA) gradient.

Forward and right-angle light scatter FCM of 23 tumor specimens revealed four main subpopulations with different size and structure. Fractionation of primary cell suspensions on a BSA gradient at unit gravity separated debris, small cells and large cells.

DNA FCM of the enriched populations demonstrated a relation between large cells and DNA aneuploidy. Epithelial cells, as recognized by cytokeratin antibodies, were also related with large cells.

The results demonstrated the usefulness of light scatter, DNA, and cytokeratin analysis of crude and fractionated tumor cell suspensions for assessment of the efficacy of a particular dispersion technique and to obtain information of the cell subsets dispersed.

Key terms: Flow cytometry, human head and neck tumors, cell size, DNA, cytokeratin

The most critical step in the sequence of investigations on primary tumor cells is the dispersion of solid tumor specimens into single, viable cells (2–4,6). The heterogeneity of human tumors with respect to malignant cell populations with different DNA content and the presence of normal cells, hampers research on tumor cells (1,8). It is therefore imperative to know exactly the type of cells that is brought into suspension before proceeding to any further investigation, like sensitivity tests to cytostatic drugs.

Flow cytometric (FCM) analysis of cell size, DNA, RNA, protein, cell surface receptors, and nuclear antigen enables the unraveling of the diversity of cell subtypes present in human tumors (1,2,5,6,12). Light scatter FCM analysis of cells in suspension can discriminate cell subsets according to cell size (forward light scatter) and cell structure (right-angle light scatter) and, as is already shown for hematopoietic cells, is a useful tool in addition to DNA analysis (5,12). Ramaekers et al. (10) have shown that, by means of cytokeratin antibodies, both the malignant and nonmalignant epithelial subset can be separated from all other cells present in suspension. The aforementioned FCM parameters in combination with fractionation of the primary cell suspension on density gradients will extend our knowledge of the cell types present in heterogeneous tumors (9,11,13).

The results of light scatter, DNA, and cytokeratin FCM of cell suspensions from human head and neck tumors are described, performed on the primary cell suspension or after fractionation by velocity sedimentation at 1g on low-density bovine serum albumin (BSA) gradients (7).

MATERIALS AND METHODS

Tumor Dispersal

Fresh tumor tissue, obtained directly after surgery, was processed aseptically as described elsewhere (2). Briefly, tumor specimens were sliced (1-mm³ pieces) with a scalpel and washed twice in Hank's balanced salt solution. The slices were divided at random and were suspended in minimal essential medium (MEM) containing enzymes, 0.1 μg/ml ketoconazole (Janssen Pharm.),

This work was supported by grant 4683 from the University of Nijmegen Research Pool (UOP) and in part by the Aank van Vlaasten Foundation.

Address reprint requests to J. Th. Bijman, St. Radboud University Hospital, Department of Internal Medicine, Division of Medical Oncology, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, the Netherlands.
Cell Size, DNA, and Cytokeratin Analysis of Human Head and Neck Tumors by Flow Cytometry

J.Th. Bijman, D.J.Th. Wagener, J.M.C. Wessels, P. van den Broek, and F.C.S. Ramaekers
Department of Internal Medicine, Division of Medical Oncology (J.Th.B., D.J.Th.W.), Division of Hematology (J.M.C.W.), Department of Otolaryngology (P.v.d.B.), Department of Pathology (F.C.S.R.), St. Radboud University Hospital, 6525 GA Nijmegen, the Netherlands

Received for publication May 21, 1985; accepted August 4, 1985

Cell subsets have been discriminated in cell suspensions derived from 37 human head and neck tumors by means of light scatter, DNA, and cytokeratin flow cytometry (FCM). Cell dispersion was performed overnight at 4°C in two different enzyme mixtures, i.e., trypsin/dithioerythritol and collagenase/DNase, under slight agitation of sliced tumor tissue. Cells were examined before and after fractionation on a discontinuous low-density bovine serum albumin (BSA) gradient.

Forward and right-angle light scatter FCM of 23 tumor specimens revealed four main subpopulations with different size and structure. Fractionation of primary cell suspensions on a BSA gradient at unit gravity separated debris, small cells and large cells. DNA FCM of the enriched populations demonstrated a relation between large cells and DNA aneuploidy. Epithelial cells, as recognized by cytokeratin antibodies, were also related with large cells.

The results demonstrated the usefulness of light scatter, DNA, and cytokeratin analysis of crude and fractionated tumor cell suspensions for assessment of the efficacy of a particular dispersion technique and to obtain information of the cell subsets dispersed.

Key terms: Flow cytometry, human head and neck tumors, cell size, DNA, cytokeratin

The most critical step in the sequence of investigations on primary tumor cells is the dispersion of solid tumor specimens into single, viable cells (2–4,6). The heterogeneity of human tumors with respect to malignant cell populations with different DNA content and the presence of normal cells, hampers research on tumor cells (1,8). It is therefore imperative to know exactly the type of cells that is brought into suspension before proceeding to any further investigation, like sensitivity tests to cytostatic drugs.

Flow cytometric (FCM) analysis of cell size, DNA, RNA, protein, cell surface receptors, and nucleolar antigen enables the unraveling of the diversity of cell subsets present in human tumors (1,2,5,6,12). Light scatter FCM analysis of cells in suspension can discriminate cell subsets according to cell size (forward light scatter) and cell structure (right-angle light scatter) and, as is already shown for hemopoietic cells, is a useful tool in addition to DNA analysis (5,12). Ramaekers et al. (10) have shown that, by means of cytokeratin antibodies, both the malignant and nonmalignant epithelial subset can be separated from all other cells present in suspension. The aforementioned FCM parameters in combination with fractionation of the primary cell suspension on density gradients will extend our knowledge of the cell types present in heterogeneous tumors (9,11,13).

The results of light scatter, DNA, and cytokeratin FCM of cell suspensions from human head and neck tumors are described, performed on the primary cell suspension or after fractionation by velocity sedimentation at 1g on low-density bovine serum albumin (BSA) gradients (7).

MATERIALS AND METHODS

Tumor Dispersal

Freh tumor tissue, obtained directly after surgery, was processed aseptically as described elsewhere (2). Briefly, tumor specimens were sliced (1-mm² pieces) with a scalpel and washed twice in Hank's balanced salt solution. The slices were divided at random and were suspended in minimal essential medium (MEM) containing enzymes, 0.1 µg/ml kethaconazole (Janssen Pharm.),

This work was supported by grant G4/83 from the University of Nijmegen Research Pool (UOP) and in part by the Aink van Vlietinger Foundation.

Address reprint requests to J. Th. Bijman, St. Radboud University Hospital, Department of Internal Medicine, Division of Medical Oncology, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, the Netherlands.
and 50 µg/ml gentamycin (Boehringer Mannheim). The enzymes and additive selected for this study were trypsin (0.25 mg/ml, type III, 11,000 BAEE U/mg, Sigma), dithioerythritol (3 mg/ml, Sigma), collagenase (2 mg/ml, type II, 132 U/mg, Cooper Biomedical), DNase (0.02 mg/ml, type I, 2400 Kunitz U/mg, Sigma), and RNase (0.06 mg/ml, type IA, 76 Kunitz U/mg, Sigma). The two dispersal mixtures were trypsin/dithioerythritol (TD) and collagenase/DNase (CD/D). Tumor tissue was incubated overnight at 4°C on a tilting table. The suspensions were centrifuged for 10 min at 200g and the pellet was resuspended in MEM plus 10% fetal bovine serum (PBS, Gibco, Grand Island, NY). The suspensions were then incubated at 37°C for 20 min and were agitated on a "Vortex-mixer" during this period for 10 s every 5 min. Afterwards, any undissociated tumor tissue was allowed to settle in the tube for 5 min at unit gravity. The resultant supernatant containing single cells and small clumps was aspirated and spun down at 200g for 10 min. Cell yield was determined, viability was evaluated by trypan blue exclusion, and dispersal capacity of the enzyme mixtures was determined (Table 1).

Flow Cytometric Analysis

Fluorescent monodisperse microspheres (diam = 1.96 µm, Polysciences Inc., Warrington, PA) were run in a flow cytometer with a Typhoon V5.0 (Amersham, Westwood, MA). Forward light scatter (FS) and side scatter (SS) were used for the analysis of cellular size and granularity. The images were acquired using a 488 nm laser (Spectra Physics) and analyzed using a multicycle algorithm. The data was then plotted on a histogram showing the percentage of cells that fall within each channel.

Table 1

| Table 1: Summary of Enzymatic Dispersal and FCM Parameters of 37 Human Head and Neck Squamous Cell Carcinomas |
|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Tissue source | Best dispersal (CD/D) | Equal | TD | DI=1 | DI>1<sup>a</sup> | FS vs RAS: Large cells<sup>b</sup> | BSA gradient enrichment<sup>c</sup> |
| Floor of mouth | 4 | 3 | 1 | 3 | 2 | 1 | 1 |
| Hypopharynx | 2 | 1 | 1 | 2 | 1 | 1 | 1 |
| Larynx | 15 | 4 | 4 | 9 | 6 | 6 | 6 |
| Lymph node | 6 | 5 | 1 | 2 | 4 | 2 | 1 |
| Mandible | 7 | 3 | 3 | 1 | 1 | 1 | 1 |
| Tongue | 3 | 2 | 1 | 2 | 1 | 1 | 1 |

<sup>a</sup>Judged by number of cells released, viability, and amount of debris (see 2).

<sup>b</sup> Number of tumors with which enrichment has been achieved.

<sup>c</sup> Detected in the TD suspension.

<sup>2</sup>Population, Figure 1.
RESULTS

Representative FCM data of five human head and neck tumors illustrating the difference in dispersal between a CDse and TD mixture are described below.

Light Scatter and DNA FCM

In Figure 1 are shown two dual-parameter (RAS vs. FS) measurements of, respectively, the CDse and TD suspension of a moderately differentiated squamous cell carcinoma (SCC) of the larynx. Four main groups were discernable with FS, as is shown in the left upper part of each panel. Population 1 represents mainly debris. The same typical contour and profile plots shown in Figure 1 were obtained with the CDse and TD suspension of a moderately differentiated SCC of the mandible (results not shown). The TD suspension contained twice the amount of large cells (population 4) as compared to the CDse suspension, while the CDse suspension contained predominately small cells (population 2 and 3). In 23 out of 37 tumor specimens, these four typical populations were detectable (Table 1). In the remaining 14 tumor specimens, only populations 1, 2, and 3 were found.

The DNA histograms of the cell suspensions from the larynx tumor showed only cells with a normal DNA content (results not shown). However, as is shown in Figure 2, the TD suspension from the tumor of the mandible contained two abnormal DNA stemlines, which were not detectable in the CDse suspension. It suggested a relation between DNA aneuploidy and size/structure of the cells.

BSA Gradient Enrichment

An extension of the aforementioned relation of light scatter properties of cells and their DNA content is shown in Figure 3. The CDse suspension of a moderately well differentiated SCC of the floor of the mouth contained a high amount of debris (Fig. 3A). Phase contrast microscopy revealed, besides debris, small cells as well as large cells. The suspension was therefore layered on a discontinuous low-density BSA gradient and five fractions of 1 ml were examined with FCM using the two light scatter parameters. The 0% fraction (Fig. 3B) contained solely debris; the 1% fraction (Fig. 3C) contained debris and small, round cells (erythrocytes, lymphocytes); the 2% fraction (Fig. 3D) contained less debris, small cells, and some larger cells (erythrocytes, lymphocytes, histiocytes, fibroblasts and small tumor cells); the 3% but especially the 4% fraction (Fig. 3E–F) contained predominantly large cells (histiocytes, tumor cells), although some small cells and debris were still present.

The light scatter parameters of the enriched subpopulations (Fig. 3B–F) are related with the DNA content of the cells, as is shown in Figure 4. The crude CDse suspension (Fig. 4A) gave a histogram with high CV and much fluorescent debris. Going from Figure 4B to 4F the fluorescent debris disappeared, while gradually cells with normal DNA content appeared, followed by an enhancement of the number of cells with DNA aneuploidy (3.1C). Comparable results were obtained with 12 other tumor specimens (Table 1). Both the number of large cells (population 4) and the number of cells with DNA aneuploidy were considerably increased (Table 1).

Cytokeratin FCM

Figure 5 shows two dual-parameter measurements of cytokeratin vs. DNA content of cells from the CDse and TD suspension of a moderately differentiated SCC of the larynx. A. CDse suspension. B. TD suspension. In each panel, the single parameters of right-angle light scatter (RAS) and forward light scatter (FS) are shown respectively in the lower right part and upper left part. A contour (64 × 64 channels) and profile plot (three dimensional) are shown respectively in the upper right part and lower left part. Debris (population 1) and three cell populations with distinct scattering properties can be distinguished. For each dual parameter measurement, 50,000 signals were processed.
larynx. Light scatter measurements indicated four times more large cells (population 4, Fig. 1) in the TD suspension compared to the CD3e suspension, while DNA FCM of the cell suspension only demonstrated cells with normal DNA content (results not shown). The TD suspension, however, contained 90% cytokeratin-positive cells, 20% of which were extremely fluorescent, while the CD3e suspension contained only 40% cytokeratin-positive cells, 50% of which were extremely positive.

Similar results were obtained with five other tumors. In one of these tumors, a moderately differentiated SCC of the larynx, an abnormal DNA stemline was detected with DNA FCM (Fig. 6A,B). The TD suspension contained twice the amount of cells with DNA aneuploidy compared to the CD3e suspension. In Figure 6C, it is demonstrated that only the abnormal DNA stemline (3.6c) stained positive for cytokeratin, indicating that no epithelial cells with normal DNA content were present in this tumor.

**DISCUSSION**

Several reports indicate that the dispersal procedure used for solid tumor tissue has a major impact on the type of cells that is brought into suspension (2–4,6). Multiparameter light scatter FCM offers the possibility to discriminate cell subsets in heterogeneous tumor suspensions (Fig. 1). In fact, measurement of RAS vs. FS enables us to distinguish four categories: 1) cellular remnants and other debris, 2) erythrocytes, 3) lymphocytes, histiocytes, fibroblasts, and small tumor cells, and 4) large histiocytes and large tumor cells. These measurements allow the quantitation of different cell populations discernable with light microscopy after routine

---

**Fig. 2.** DNA content distribution (1,024 channels) of a moderately differentiated SCC of the mandible. A. CD3e suspension, B. TD suspension. Cells with 2c-DNA content are located in channel 300.

**Fig. 3.** Light scatter analysis (RAS vs. FS) of the CD3e suspension of a moderately well differentiated SCC of the floor of the mouth. A, profile plot of the crude suspension, indicating an enormous amount of cellular remnants and extracellular debris. B–F, separation of debris from small and larger cells on a discontinuous low density BSA gradient, showing the 0, 1, 2, 3, and 4% BSA fractions, respectively. For each dual parameter measurement, 50,000 signals were processed.
staining by the May Grunwald-Giemsa or Papanicolaou methods. Furthermore, light scatter FCM is an elegant method to test the effect of density gradients on cell separation, as is shown in Figure 3. Enrichment of abnormal DNA stemcell populations on low-density BSA gradients improved the CV of the DNA histograms (Fig. 4A vs. Fig. 4F), illustrating that damaged cells may also be separated from intact cells.

A comparison of the data shown in Figures 3 and 4 indicated a relation between cell size and DNA aneuploidy. Better dispersal of cells with DNA aneuploidy with a TD mixture compared to a CD3e mixture could also apply to epithelial tumor cells with a normal DNA content. As demonstrated in Figure 1, the CD3e and TD suspension contained three distinct cell subsets (populations 2, 3, and 4). Only the relative number of cells in each population was varying. It is therefore conceivable that the high number of large cells (population 4) could suggest more tumor cells with normal DNA content. The data displayed in Figures 5 and 6 substantiated this hypothesis. They clearly demonstrated that a TD mixture suspends more epithelial cells from tumor tissue as compared to the CD3e mixture.

In conclusion, measurement of light scatter properties of cells from solid tumor specimens provides quantitative information concerning the subpopulations present.

Size and structure parameters are correlated with DNA aneuploidy, which could be extended to tumor cells with normal DNA content by means of cytokeratin FCM. Fractionation of the cell suspensions on low-density BSA gradients improved the quality of the cell suspensions and enriched for tumor cells. Presently, investigations are aimed at designing a serum-free medium formulation, which specifically will support the in vitro growth of primary squamous carcinoma cells in a monolayer culture system. Cell types emerging in vitro will be compared with the cell types present in the primary cell suspensions using FCM.

![Diagram](image-url)

**Fig. 4**. DNA content distribution (1,024 channels) of the crude CD3e suspension and its separated fractions shown in Figure 3. Cells with 2c-DNA content are located in channel 300.

**Fig. 5**. Dual-parameter measurement (64 x 64 channels) of cells from a moderately differentiated SCC of the larynx stained with propidium iodide (ordinate) and FITC-indirect immunofluorescence of cytokeratin (abscissa). A. CD3e suspension. B. TD suspension. Propidium iodide and FITC-positive cell populations are indicated by a window. For each dual-parameter measurement, 50,000 signals were processed.
ACKNOWLEDGMENTS

The authors are indebted to H. Mann, MD, and I. Brussas, MD, for providing tumor specimens and for their cooperation in a rapid processing after surgical resection. They also wish to express their gratitude to Prof. Dr. C. Haansen, Dr. P. de Mulder, Dr. B. de Pauw, and Dr. W. Kuypers for helpful discussions. Hans Beck is acknowledged for his excellent help with the cytokeratin determinations.

LITERATURE CITED


