DNA cytometry of musculoskeletal tumors

A review

Andris Kreicbergs

Quantitative DNA analysis has become increasingly applied over the last decade for characterizing tumors as an adjunct to histopathologic assessment. (For review, see Andreeff 1986, Johnson 1988, Laerum 1981, Tribukait 1981). In several tumor entities, the discrimination between lesions with a normal and abnormal DNA content has been shown to provide prognostic and/or diagnostic information (for review, see Atkin and Kay 1979, Barlogie et al. 1985, Friedlander 1984 et al.).

Cellular DNA content

Normal tissue

The normal human cell is characterized by a constant DNA content corresponding to 46 chromosomes. It is referred to as euploid or diploid. The latter term denotes that the cell contains a double set of 23 chromosomes, as opposed to a single set in the germ cell. Exceptions to the DNA constancy can be encountered in certain tissues, such as liver, where the cells may exhibit the double amount, then by definition being tetraploid (4 x 23 = 92 chromosomes). Cells with a DNA content corresponding to multiples of 23 chromosomes, i.e., 46 or 92, are denoted euploid. Notably, a chromosomal arrangement of DNA is only observed during cell division, i.e., mitosis. Thus, the vast majority of cells in any tissue contains DNA in dispersed form, not permitting identification and the counting of chromosomes.

In normal tissue, deviations from euploidy are only seen in proliferating cells. To replicate, the cell is triggered by unknown mechanisms from a quiescent state (Go-phase) into the cell cycle defined by the G1-, S- and G2-phases (Figure 1). During the G1-phase, the cell nucleus increases in size mainly by influx of proteins from the cytoplasm, but remains diploid. In the S-phase, synthesis of DNA is initiated and continues until the DNA content is doubled. Over this relatively short period, the cell will display a successively increasing amount of DNA, then by definition being noneuploid until replication is completed. The ensuing G2-phase is characterized by a tetraploid cell growing in size and preparing for cell division. Completion of the cell cycle occurs when the premitotic G2 cell, having entered the mitotic (M) phase, splits into two cells, each with exactly the same amount of DNA as the original diploid Go cell. Because replication is a rapid and a rare event in normal tissue, few cells will exhibit a DNA content deviating from the diploid. Even in tissues with a high cell turnover, such as bone marrow and intestine, the fraction of proliferating cells rarely exceeds 15 percent.

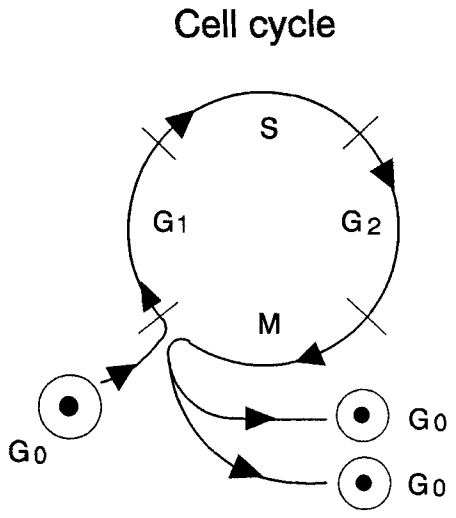

Figure 1. Diagram of the progression of a cell through the different phases of the cell cycle. G1: preparation for DNA replication, S: DNA synthesis, G2: preparation for cell division, M: mitosis. The normal cell is tetraploid during G2 and M.

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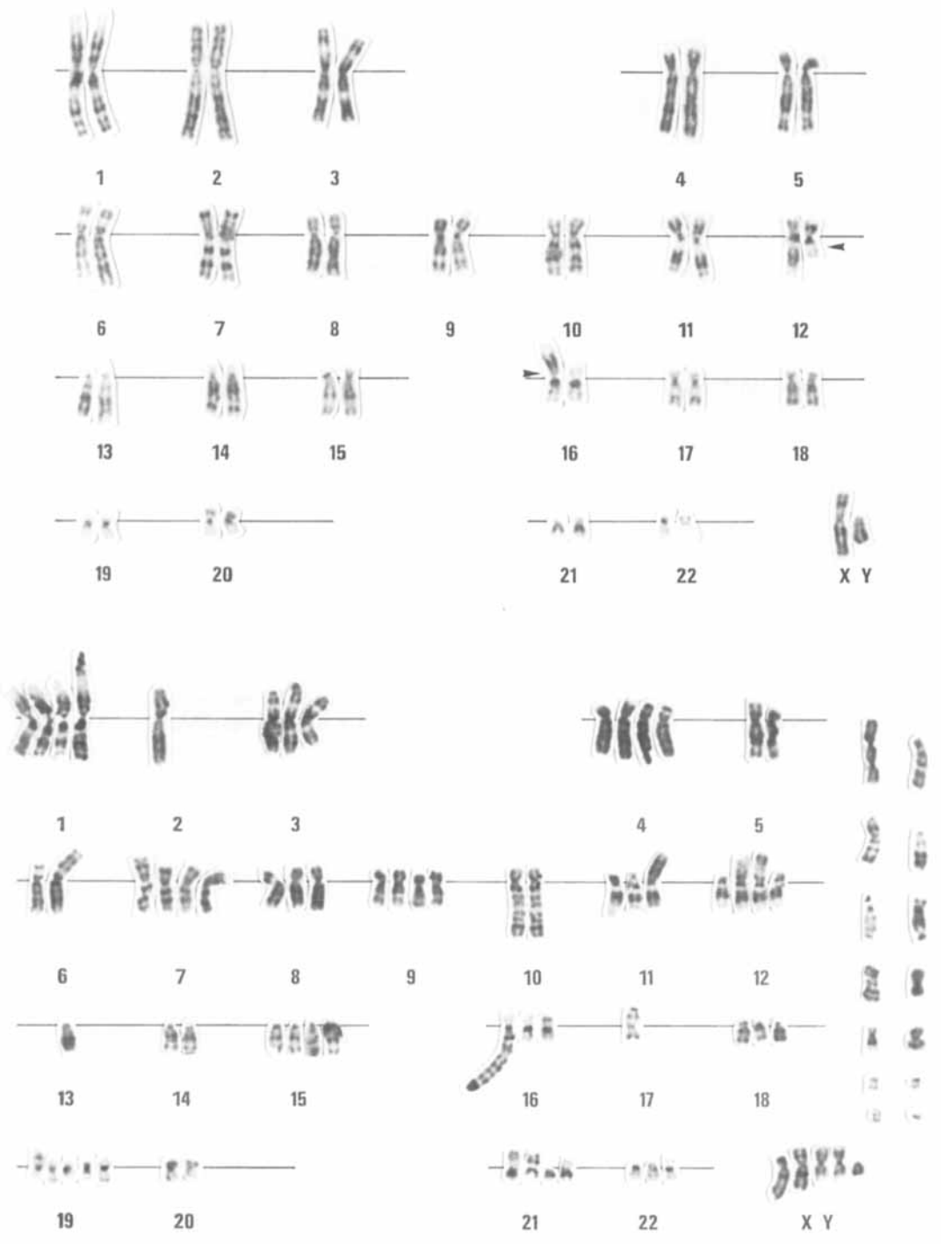

Figure 2. Cytogenetic analysis of myxoid liposarcoma cell (46 chromosomes) with a structural change: translocation between chromosome 12 and 16 (top), and malignant fibrous histiocytoma cell with both numerical (83 chromosomes) and structural changes (bottom).

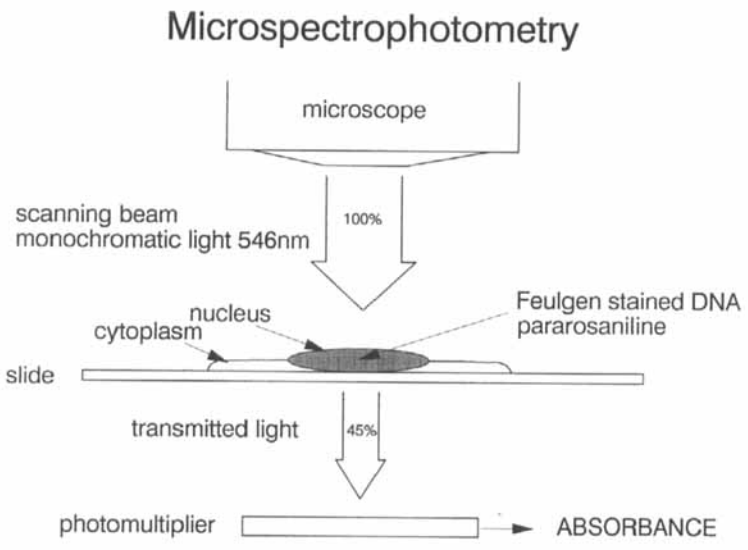

Figure 3. Schematic illustration of microspectrophotometry.

Neoplastic tissue

Cytogenetic studies have shown that neoplastic cells are characterized by structural and/or numerical chromosome changes (Heim and Mitelman 1987, Mitelman 1988, et al. 1989, Trent et al. 1988). In certain neoplasias, including solid mesenchymal tumors, specific karyotypic rearrangements have been identified (Figure 2), which indicate that these anomalies are nonrandom and thus tumor-specific. Numerical abnormalities (Figure 2), i.e., aneuploidy, are often sufficiently pronounced to permit determination by quantitative cytophotometry (Atkin et al. 1966, 1971, 1987, Böhm and Sprenger 1971, 1975 et al., Leuchtenberg et al. 1954). Notably, DNA cytometry can be applied to cells regardless of whether DNA is present in chromosomal or dispersed form, whereas karyotyping can only be applied to cells in mitosis.

A common feature in neoplasia, apart from chromosomal abnormality, is increased growth rate as reflected by an abnormally high fraction of cells in the S- and G2 + M phases of the cell cycle. Most investigators seem to agree that the fraction of proliferating cells in normal tissue at the extreme may reach 15–20 percent. Hence, values beyond 20 percent can be regarded as reflecting neoplastic abnormality.

Methodology

DNA cytometry

Cellular DNA analysis is based on determining either absorbance or fluorescence of dyes specifically bound to DNA. Once the amount of dye is determined, the DNA content of the cell can be deduced. However, the methods available do not permit quantitation of the absolute amount of DNA. Therefore, comparison to the amount of DNA stain in normal control cells, e.g., lymphocytes or fibroblasts, is a prerequisite for determining the DNA content of tumor cells.

Basically, there are two means, both optical, for determining cellular DNA content, i.e., microspectrophotometry (MSP) and flow cytometry (FCM). The former method is applied to cells on slides and the latter to cells in suspension.

Microspectrophotometry

Caspersson (1936) introduced microspectrophotometry, which permits the quantitation of chemical constituents within a cell. Later, Caspersson and Lomakka (1970; Lomakka 1965) developed the scanning microspectrophotometer. The technique is based on determining the amount of monochromatic light passing through an object (Figure 3). The wave length of the scanning beam is chosen according to maximum absorption by the dye specifically bound to the substance of interest. The light transmitted is

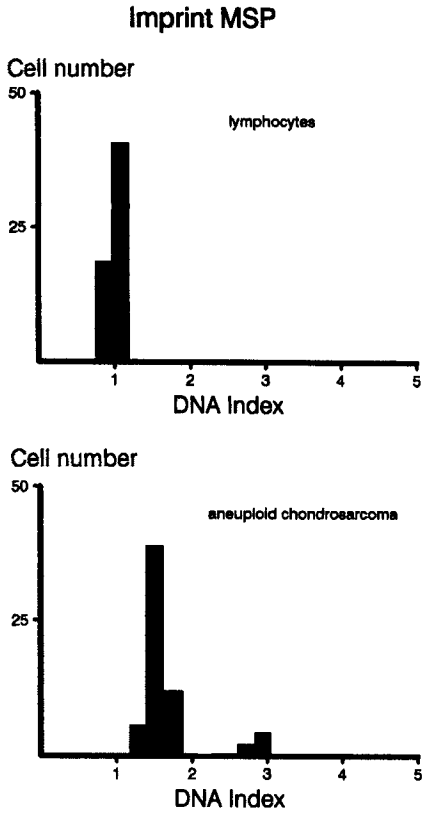

Figure 4. DNA histogram of an osteosarcoma with a peak DNA value deviating from that of normal lymphocytes.

quantitated by means of a photomultiplier. Once the transmittance has been assessed, the absorbance can be derived according to the Lambert-Beers law, which states that the amount of light transmitted through a homogeneous solution depends on the concentration. Absorbance is inversely and exponentially related to transmittance. Pararosaniline is the absorbing DNA dye mostly used for MSP according to a staining procedure described in the early 1920s by Feulgen and Rossenbeck 1924). For review, see Caspersson (1979).

MSP measurements are applied to cells on slides under visual control. Routinely, 10–50 normal cells, e.g., lymphocytes or fibroblasts, are analyzed to obtain the diploid reference value, defined by the mean absorbance of these cells. Subsequently, 100–200 tumor cells are morphologically screened and measured. The DNA absorption values of the normal cells and tumor cells are plotted in frequency histograms for comparison (Figure 4). If the distribution of the tumor cell DNA values differs from that of the normal cells, the tumor is characterized as nondiploid, i.e., tetraploid or aneuploid.

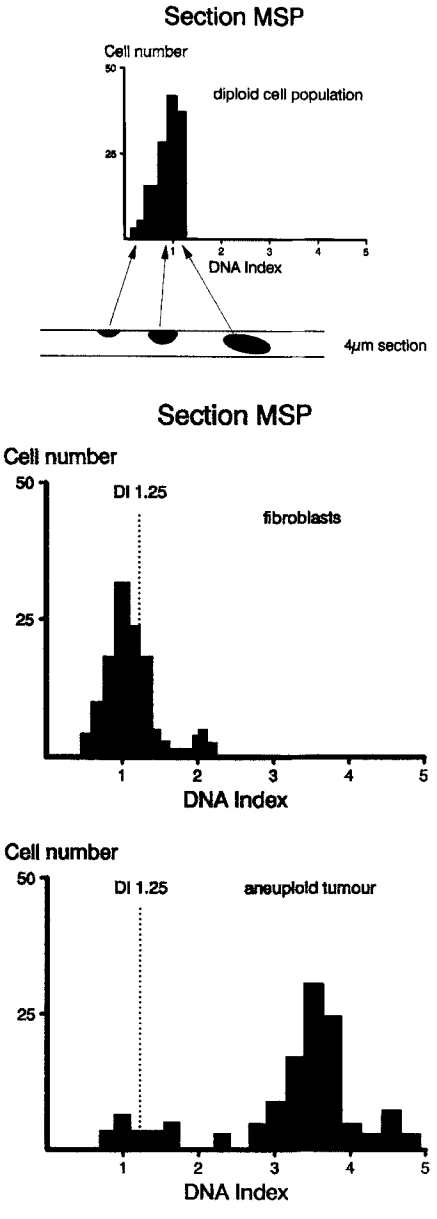

Figure 5. DNA histogram of a) normal cells analyzed in tissue section (4 μm). The true diploid value is represented by the unsectioned cell, b) tumor cells with DNA values deviating from those of normal cells in the same section.

MSP can be applied both to imprint preparations (whole nuclei) and to sections (partly cut nuclei) from paraffin-embedded specimens. Although MSP of sections (4–5 μm) is associated with a methodologic error, i.e., measurement of a substantial number of cut nuclei (Figure 5), it still provides valid data (Bauer et al. 1986, Kreicbergs et al. 1980, et al.

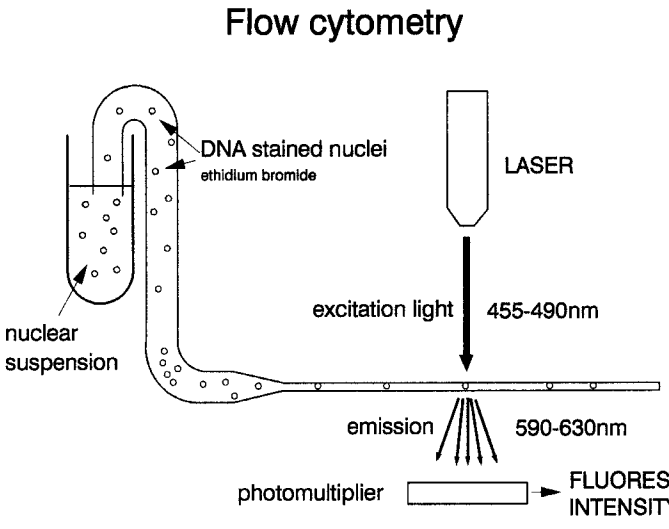

Figure 6. Schematic illustration of flow cytometry.

1981, Bennington and Mayall 1983, McCready et al. 1983, Moberger 1989). The major advantage is that it offers DNA analysis of tissue with preserved architecture. Thus, different tissue areas of interest can be selected for measurement. Moreover, it permits retrospective DNA studies of old archival specimens, which have been shown to retain adequate Feulgen DNA stainability (Bauer and Kreicbergs 1987, Kreicbergs et al. 1980, Moberger 1989).

An alternative to slide measurement of nuclear DNA absorbance by microspectrophotometry is determination of DNA fluorescence by cytofluorometry (Böhm and Sprenger 1968, 1971). So far, the latter method has not gained wide application due to methodologic problems, such as fading of fluorescence (Bjelkenkrantz 1983) at slide analysis. With the recent development of techniques for rapid slide cytofluorometry, the method may become more widely used.

Flow cytometry

The flow cytometer (FCM) was introduced by Crossland-Taylor (1953) and Coulter (1956), and further developed by Kamentsky et al. (1965) and Van Dilla et al. (1969). FCM has become increasingly applied over the past 15 years at the expense of MSP, because it offers higher speed and resolution. Suspensions of cells or nuclei are prepared from solid tissue by mechanical and chemical disaggregation. After cell fixation, a fluorescent DNA dye is added to the suspension. The dyes most commonly used either bind specifically to adenine-thymine or intercalate into double-stranded nucleic acids. Because the latter dyes, e.g., ethidium bromide and propidium iodide, also stain RNA, the preparations must be treated in RNase before DNA measurement. For an extensive survey of different techniques and applications of flow cytometry see Melamed et al. (1979).

The essence of FCM has been described as the bringing of suspended cells one by one to a detector by means of a flow channel (Figure 6). The cell suspension is injected into a faster flowing stream of fluid, which provides a laminar sheath around the particle stream to align and center the cells (hydrodynamic focusing). The flow cytometer is commonly equipped with a laser or a mercury lamp, which

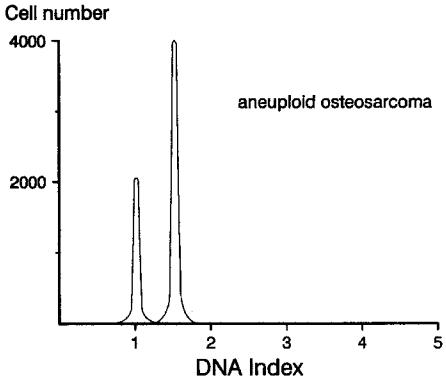

Figure 7. Schematic flow DNA histogram of osteosarcoma showing a typical aneuploid peak to the right of the diploid peak.

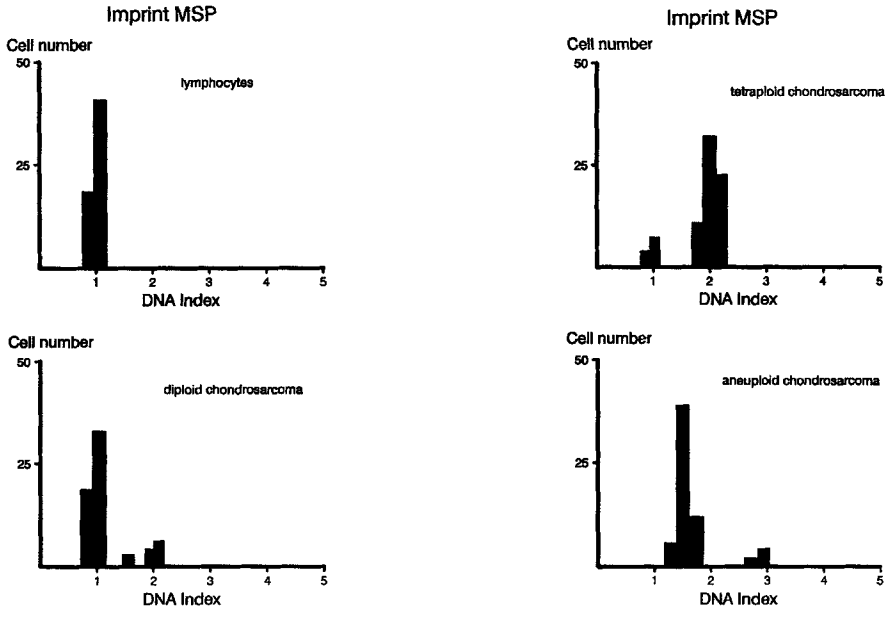

Figure 8. DNA histograms of diploid (DI 1.0), tetraploid (DI 2.0), and aneuploid (DI 1.6) chondrosarcomas.

generates a beam of appropriate wave length. As the cells pass through the narrow flow channel, they are hit, one by one, by the focused beam at a determined angle. When hit by the excitation light, fluorescence is emitted from each cell and recorded by a photomultiplier. The fluorescence intensity of each cell reflects its DNA content. More than 20,000 cells can be analyzed within a couple of minutes. The recorded fluorescence intensities are computerized and presented in a frequency DNA histogram of all cells analyzed (Figure 7). Human lymphocytes, either measured separately or added to the cell suspension, are commonly used as controls to obtain the diploid reference value. Alternatively, trout or chicken red cells with a well-established DNA content relative to that of human diploid cells may be used as standards. (For further details see Göhde et al. 1979, Traganos 1984, Vindeløv et al. 1983).

For clinical purposes, FCM was initially applied to fresh samples from patients with hematologic malignancies, from which cell suspensions were easily prepared (Barlogie et al. 1976, et al. 1980). Later, it proved feasible to obtain cell suspensions also from solid tumors (Barlogie et al. 1978, Ensley et al. 1987, Pallavicini et al. 1978, Slocum et al. 1981, Thornwaite et al. 1980, Vindeløv 1977, et al. 1983). However, the procedures utilized still vary extensively in attempts to improve chemical and mechanical disaggregation of solid tumors.

Hedley et al. (1983) were the first to report preparation of cell suspensions from archival paraffin-embedded specimens by dewaxing and subsequent disaggregation. Although the technique has yet to be further developed, it has the potential of permitting retrospective DNA studies by FCM. The retrospective approach had previously been confined to MSP of tissue sections. In a recent review (Hedley 1989) of more than 100 studies using the procedure, only one dealt with orthopedic tumors (Radio et al. 1988).

Data interpretation

In a technical sense, MSP and FCM are designed to determine the DNA content of individual cells. However, the purpose is to collect data for ploidy classification of a whole cell population. Principally, ploidy classification of a tumor is based on the most prominent peak (modal DNA value) of the histogram. According to current terminology (Hiddeman et al. 1984), the modal value is denoted as the DNA Index (DI) of the tumor. Basically, three ploidy types may be encountered, i.e., diploid (DI = 1.0), tetraploid (DI = 2.0), and aneuploid (other DI) tumors (Figure 8). However, within the same tumor cell population a mixture of cells exhibiting differ-

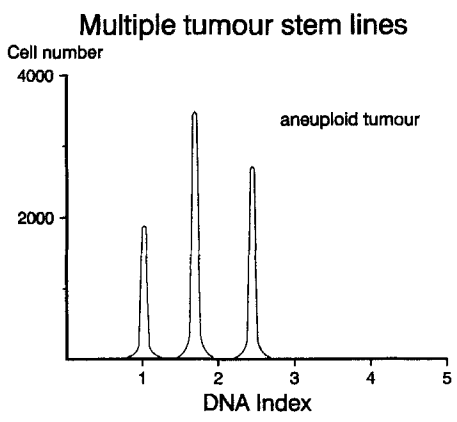

Figure 9. Schematic flow DNA histogram of a tumor with two aneuploid peaks reflecting DNA heterogeneity.

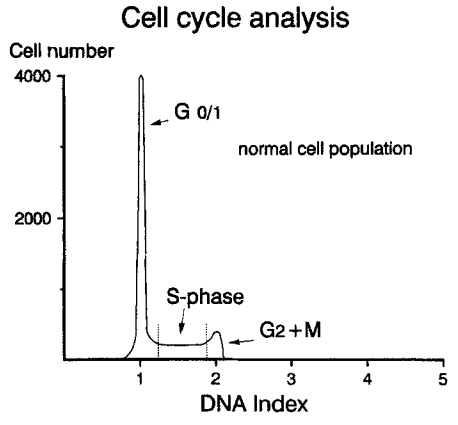

Figure 10. Schematic DNA histogram of proliferating normal cells. Note the overwhelming predominance of cells in the Go/1-phase, i.e., diploid cells.

ent DNA values is occasionally seen, reflecting cytochemical (DNA) heterogeneity. Although there may be widely scattered values, most heterogenic tumors display a bimodal DNA distribution (Figure 9). Thus, an aneuploid lesion may contain two aneuploid cell lines, e.g., DI 1.3 and DI 2.5, or a diploid subpopulation, e.g., DI 1.0 and DI 1.3.

Interpretation of data differs in some decisive respects between MSP and FCM. The former method is applied to cells on slides allowing for morphologic screening of different cell types. Normal and neoplastic cells can be measured separately to obtain *two* histograms for comparison. Quite differently, FCM is based on analysis of suspended cells that represent a random mixture of normal and neoplastic cells. Hence, the technique provides only *one* single histogram depicting the combined distribution of DNA values of normal and neoplastic cells; the proportion of each cell type remains unknown. The peak with the lowest DNA value is supposed to reflect the diploid DNA value (D = 1.0) based on the assumption that a tumor specimen always contains an admixture of normal diploid cells. The presence of an additional peak, which is aneuploid, can with certainty be considered to represent tumor cells. However, a problem of interpretation arises when the histogram displays only a diploid peak or a diploid-tetraploid (DI 1.0–2.0) curve. Such distributions presumably reflect a mixture of normal and neoplastic cells, but could also represent exclusively normal cells. Hence, in such instances the representativeness of the analyzed "tumor" specimen can be questioned.

As mentioned, MSP can be applied to both imprint preparations and tissue sections. The latter approach has the advantage of permitting selection of different pertinent tissue areas for analysis. On the other hand, it is associated with the methodologic error of measuring a substantial number of sectioned nuclei. Although this decreases the accuracy in determining the DNA content of individual nuclei, it does not seem to affect ploidy classification of a tumor cell population in a decisive way, i.e., the discrimination between diploid and nondiploid tumors (Bauer 1986, Kreicbergs 1980 et al., 1981, Bennington and Mayall 1983, Moberger 1989). Several means of interpreting histograms from tissue section measurements have been proposed in attempts to define ploidy classes taking into account the inevitable variation of DNA values caused by sectioning (Bauer et al. 1986, Bennington and Mayall 1983, Kreicbergs et al. 1980, McCready and Papadimitriou 1983). Basically, the procedure can only be used to determine whether a tumor is diploid or hyperploid, the latter term denoting an abnormally increased DNA content. Hence, MSP does not permit the assessment of the modal DNA value (DI) of an aneuploid lesion. Nor can it be used to discriminate reliably between tetraploid and aneuploid tumors. These problems are not encountered when analyzing imprint preparations of unsectioned whole cells. This procedure allows for accurate determination of cellular DNA content and, hence, tumor modal DNA value. Although tissue architecture is not preserved as seen in sections, control cells and neoplastic cells can be identified for separate measurement and subsequent histogram comparison.

Although FCM occasionally entails the interpretational problem of separating different cell populations, it is preferable to MSP with respect to

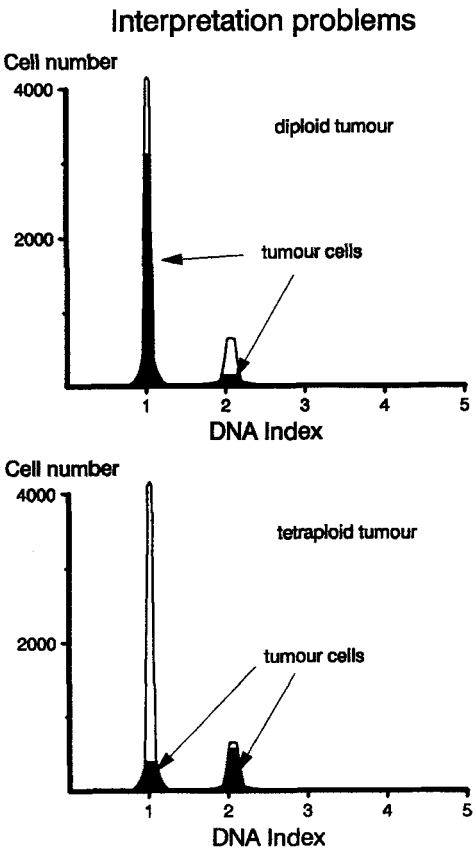

Figure 11. Diploid tumor (top) and predominantly tetraploid tumor (bottom) yielding identical DNA histograms. DNA values of normal cells—white, tumor cells—black.

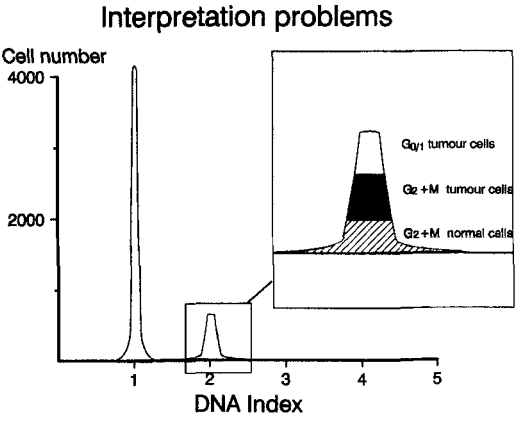

Figure 12. Schematic diploid-tetraploid FCM distribution illustrating conceivable cell subpopulations with a tetraploid DNA content. The occurrence or relative proportion of each subpopulation remains unknown.

speed and resolution; peak DNA values (DI) are more accurately determined. Moreover, it permits the assessment of the fraction of cells in different phases of the cell cycle (Baisch 1975; Figure 10). Of particular interest is the percentage of cells in the S and G2 + M phase, because it reflects the proliferative activity. This feature is presumably of clinical relevance by being an approximative descriptor of tumor growth rate. MSP of imprint preparations might also be used for cell cycle analysis. However, given the facts that MSP is a time-consuming procedure and cell-cycle analysis requires the measurement of at least 1,000 cells, the approach is not feasible for practical reasons.

Comparative studies of MSP and FCM have shown that there is a good correlation as regards gross tumor ploidy, i.e., the discrimination between diploid and nondiploid lesions (Auer 1980, Bauer 1986, Kreicbergs 1981). The main difference, apart from speed and resolution, is the uncertainty associated with FCM in assessing tumor diploidy and tetraploidy. A histogram showing only a diploid peak may be interpreted as reflecting 1) exclusively normal cells (= nonrepresentative tumor specimen) or 2) a mixture of normal and neoplastic cells (= tumor diploidy). A histogram showing a marked bimodal diploid-tetraploid distribution poses similar difficulties (Figure 11) and may be interpreted as reflecting: (1) exclusively the DNA values of normal cells, where a high proportion is in the G2 + M phase (= nonrepresentative tumor specimen); (2) a mixture of normal and neoplastic cells (= tumor diploidy), where one or both cell populations contribute to the high proportion of cells in the G2 + M phase; (3) a truly G0/G1-tetraploid tumor, where the diploid peak exclusively represents the normal cells; or (4) a heterogeneous tumor containing both diploid and G0/G1 tetraploid cells. Figure 12 illustrates the latter case, where three cell subpopulations constitute the tetraploid peak. Using FCM, the only bimodal diploid-tetraploid histogram, which with certainty can be regarded to reflect DNA values of tumor cells is one with a tetraploid peak containing more than 20 percent of all cells analyzed. Some of these tetraploid cells can safely be considered G0-tumor cells, and not merely G2 + M cells of unknown origin.

It appears that a combined approach based on both FCM and MSP offers optimal information. Whenever a diploid or a diploid-tetraploid distribution is assessed by FCM, unless > 20 percent tetraploid cells are detected, uncertainty will prevail

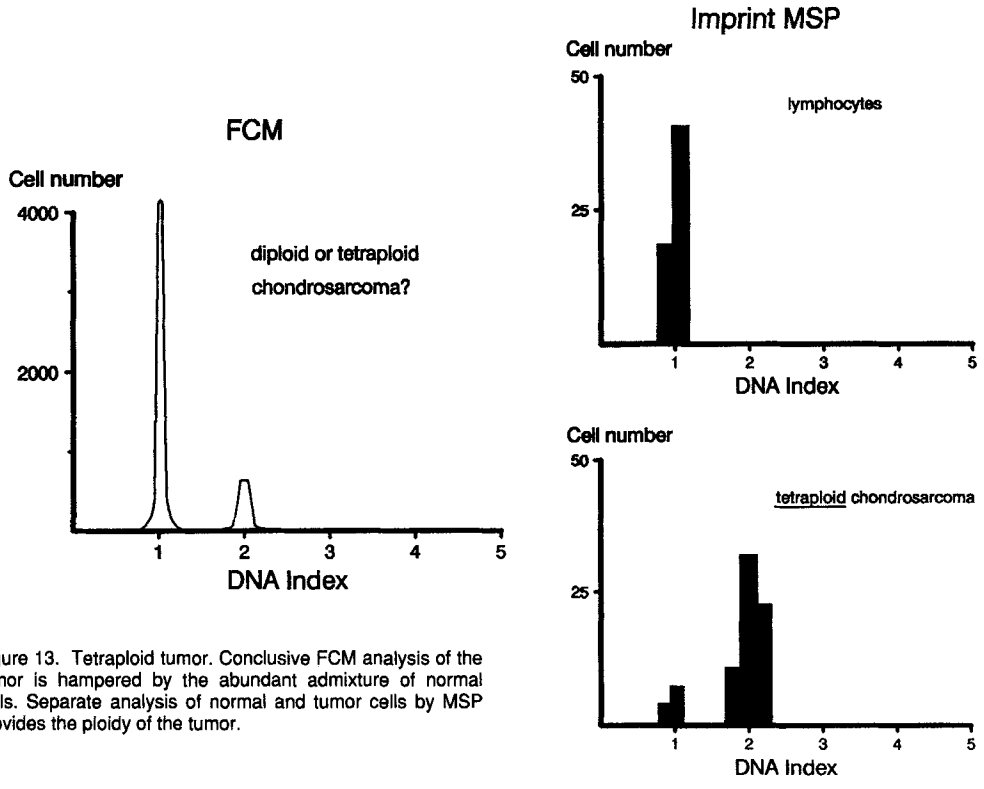

Figure 13. Tetraploid tumor. Conclusive FCM analysis of the tumor is hampered by the abundant admixture of normal cells. Separate analysis of normal and tumor cells by MSP provides the ploidy of the tumor.

about the representativeness of the analyzed specimen. In such instances, MSP of visually identified cells (Figure 13) may establish whether the results obtained by flow cytometry are valid, i.e., whether they reflect the DNA content of tumor cells, and not merely that of normal cells. On the other hand, an aneuploid peak according to FCM can safely be relied upon as representing tumor cells, and therefore precludes the need for complementary MSP.

Clinical application

An extensive number of tumor entities has been investigated by quantitative DNA analysis over the past decades. Atkin and Richards, as early as the 1950s, reported on major series of various carcinomata analyzed by MSP (Atkin and Richards 1956). With the introduction of FCM, the number of tumor DNA studies has increased dramatically. However, the application of DNA analysis to mesenchymal tumors remains limited. One reason is the rarity of these tumors; another is the difficulty in retrieving cell material from highly fibrous and osseous lesions for FCM due to a high content of dense collagen and/or mineral. In addition, demineralization of bone tumors may impede DNA stainability, sometimes to such an extent that neither FCM nor MSP can be applied (Bauer et al. 1987, Kreicbergs et al. 1980, 1981). So far, approximately 30 DNA studies of mesenchymal tumors have been published, most of which concerning bone tumors.

Bone tumors

It appears that Stich and Steele (1962) were the first to analyze a bone tumor: MSP of Feulgen-stained tissue sections disclosed that an osteosarcoma was triploid. Applying a similar methodology to seven malignant bone tumors, Födisch et al. (1975) concluded that osteosarcomas were aneuploid and chondrosarcomas near-diploid.

Cartilaginous tumors

It is well known that cartilaginous tumors pose problems in the distinguishing between benign and malignant variants and also in assessing the degree of

malignancy. This may explain why the first major DNA studies of bone tumors in 1979 and 1980 focused on cartilaginous lesions. These studies were retrospective, based on MSP of tissue sections from archival paraffin blocks (Cuvelier et al. 1979, Kreicbergs et al. 1980, 1981, et al. 1982). Basically, they showed that benign cartilaginous tumors consistently were diploid, whereas the malignant were either diploid or hyperploid. The latter finding shows that DNA analysis can not be used to discriminate between benign and malignant lesions, unless aneuploidy (hyperploidy) is detected. Among 48 chondrosarcomas analyzed (Kreicbergs 1981, et al. 1982), there was a relationship between ploidy and histologic grade. Thus, in general low-grade (I) lesions were diploid and high-grade lesions (III) hyperploid. Of particular interest, however, were the exceptions to this rule. Notably, half of the grade II chondrosarcomas were diploid and the other half hyperploid. This shows that tumors exhibiting similar histology can differ in ploidy. Hence, DNA determination offers information beyond that obtained by histologic assessment.

In a prognostic analysis of the series patients with diploid lesions were found to have a significantly higher 10-year survival rate (0.77) than those with hyperploid (0.27) (Kreicbergs 1981, et al. 1982). According to histologic grade (I–III) the corresponding 10-year survival rates were 0.64, 0.56, and 0.18, respectively. Among 14 cases with a highly aggressive clinical course, as defined by death within 2 years of diagnosis, 13 had hyperploid lesions; histologically, however, only 7 had high-grade (III) tumors. These findings indicate that ploidy level is a better predictor of clinical course in chondrosarcoma than histologic grade.

The first flow cytometric study of bone tumors was reported in 1981 (Kreicbergs et al. 1981). It included five chondrosarcomas and seven osteosarcomas. Tissue samples yielding conclusive DNA histograms were obtained by selecting soft tissue parts of the tumors. The results of FCM were checked by comparative MSP of tissue sections and imprint preparations of the same tumors. Complete agreement was found between the three methods in terms of discriminating between diploid and nondiploid tumors. This study was followed by a FCM report on 15 cartilaginous tumors by Alho et al. (1983) showing that all six benign lesions were diploid, four of five low-grade chondrosarcomas diploid and all four high-grade aneuploid. During recent years, additional FCM studies of cartilaginous tumors have been reported from four centers (Kreicbergs et al. 1984, Mankin et al. 1985, Heli et al. 1985, Xiang et al. 1987), essentially confirming the first observations.

In conclusion, collective data indicate that all benign cartilaginous tumors are diploid, whereas the malignant lesions may be either diploid or aneuploid. The clinical relevance rests upon the observation that aneuploidy, apart from precluding benignity, seems to reflect a high degree of malignancy.

Osteosarcoma

The first two DNA studies on osteosarcoma reported in the early 1980s (Kreicbergs 1982 et al., et al. 1984) concluded that probably all classical high-grade lesions, regardless of histogenetic subtype, were aneuploid, whereas paraosteal low-grade variants were diploid. These observations were based on MSP of 26 classical and two paraosteal osteosarcomas. In the other study utilizing FCM all the 16 high-grade lesions proved to be aneuploid, whereas two paraosteal lesions were diploid. Subsequently, two reports in 1985 disclosed that classical high-grade osteosarcomas occasionally are diploid. In a study by Mankin et al. (1985) diploidy was found in nine of 43 high-grade osteosarcomas, apart from the eight paraosteal variants. Heliö et al. (1985) reported diploidy in two of 15 osteosarcomas. These studies were the first to show that high-grade osteosarcomas not consistently are aneuploid. This was confirmed by Hiddemann et al. (1987) reporting diploidy in 3 of 21 cases. However, the same year Xiang et al. (1987) found aneuploidy in all 19 cases analyzed. The reason for these contrary observations may be found in the interpretation of the histograms. This applies both to the definition of tetraploidy and the distinction between diploid and near-diploid tumors. The latter lesions are regarded as aneuploid by some investigators, but as diploid by others. Finally, the discrepant findings may be explained by differences in the histologic definition of high-grade osteosarcoma.

The most comprehensive DNA study of osteosarcoma was reported in four papers by Bauer (1988, et al. 1989). It included 102 high-grade osteosarcomas and four paraosteal osteosarcomas analyzed by MSP of tissue sections. In addition, the study comprised 41 benign tumors representing entities, which sometimes are mixed up with osteosarcoma, such as osteoblastoma, aneurysmal bone cyst, fibrous dysplasia, and giant cell tumor. Finally, there were 17 other tumors that had caused diagnostic difficulties as to being benign bone tumors or osteosarcomas.

The analysis showed that 97 of 102 classical osteosarcomas were hyperploid, the four paraosteal os-

teosarcomas were diploid and all 41 benign lesions were diploid. At follow up, the exceptional cases with diploid high-grade osteosarcoma were free from disease. As to the predominant group of hyperploid cases, no specific feature of the histograms was found to provide prognostic information. Among the 17 tumors causing differential diagnostic difficulties seven were diploid and 10 hyperploid. All 7 patients with diploid lesions were free of disease at follow-up, ranging from 3 to 12 years. In contrast, in the hyperploid group there were eight local recurrences; 3 of these patients had died of tumor disease and 1 had lung metastases. Thus, in this group of 17 patients, where uncertainty had prevailed concerning the diagnosis of osteosarcoma, recurrence and death was consistently associated with hyperploidy.

In the same work, 47 osteosarcomas were also analyzed by FCM. Two tumors were diploid and 45 aneuploid; the majority exhibited a triploid peak. In 35 patients with adequate follow-up time, neither the peak DNA value nor the presence of multiple aneuploid peaks was found to be of prognostic value. However, cell-cycle analysis, feasible in 28 cases, seemed to provide prognostic information. Thus, for the 7 patients with tumors containing < 15 percent S-phase cells, the 3-year metastasis free survival rate was 0.71, compared with 0.27 for the 21 patients with > 15 percent S-phase cells. In another prognostic FCM study of osteosarcoma by Look et al. (1989), near-diploid variants of osteosarcoma were claimed to respond more favorably to adjuvant chemotherapy than other ploidy variants.

The combined findings strongly suggest that aneuploidy is a characteristic feature of high-grade osteosarcoma. Although of limited prognostic value, DNA analysis appears to be of differential diagnostic value. Whenever doubts arise about the diagnosis of osteosarcoma versus benign lesions, DNA cytometry demonstrating aneuploidy (hyperploidy) offers decisive information. Apart from the therapeutic implication, routine DNA characterization would make comparison of treatment results from different centers more meaningful. It may be questioned whether diploid lesions should be included in clinical trials on, e.g., adjuvant chemotherapy.

Giant cell tumors

Considerable interest has been focused on DNA analysis of giant cell tumors. It is well known that occasional lesions are associated with a malignant clinical course, which is commonly not possible to predict by histologic evaluation. So far, the total number of lesions analyzed by different groups approaches 100 (Kreicbergs et al. 1984, Bauer 1988, Mankin et al. 1985, Heli 1985, Xiang et al. 1987, Ladanyi 1989). Only two tumors have been found to be clearly aneuploid, although Xiang et al. (1987). reported that as many as 16 of 18 lesions had an abnormal near-diploid pattern. Mankin et al. (1985) in a series of 46 tumors reported that 1 exhibited a small (5.2 percent of the cells) aneuploid peak and 17 a tetraploid (>11 percent of the cells) peak. Another two studies including 21 tumors showed that all were diploid (Bauer 1988, Heli et al. 1985). In a recent study by Ladanyi et al. (1989) based on FCM of disaggregated paraffin-embedded specimens from seven metastasizing giant cell tumors five were diploid, one was tetraploid, and only one was aneuploid. The combined data, despite prevailing differences in histogram interpretation (see Xiang et al. 1987), seem to indicate that the vast majority of giant cell tumors are (near-) diploid. Many lesions contain a high proportion of tetraploid cells presumably reflecting increased proliferative activity. These findings comply with the benign, but aggressive growth pattern of giant cell tumors. However, from the study of Ladanyi et al. (1989) it appears that DNA analysis in giant cell tumors fails to provide information about the risk of metastatic spread.

Miscellaneous malignant bone tumors

DNA data on other primary bone tumors are too limited to permit any conclusions. In Ewing's sarcoma preliminary data indicate that diploidy is not uncommon, despite high malignancy (Mankin et al. 1985). This finding complies with cytogenetic data; most Ewing's sarcomas have a balanced translocation between chromosomes 11 and 22 (Turc-Carel et al. 1988). In this context, it should be noted that ploidy determination in other round cell tumors such as lymphoma appears to be of limited predictive value (Christensson 1983). Instead, assessment of cycling (S- and G2- + M-phase) cells has proved to be of prognostic relevance (Barlogie et al. 1983, Christensson 1983, Shackney and Skramstad 1979). Whether this also applies to Ewing's sarcoma has not been established.

Summary

The most consistent finding from DNA analysis of primary bone tumors is that not a single case of

aneuploidy has been encountered among clearly benign lesions. Although diploidy in benign tumors may seem self-evident, it represents a feature of decisive importance by providing objective support for histologic diagnosis. Conversely, and still more importantly, aneuploidy precludes benignity. Moreover, it seems that aneuploid bone tumors are highly malignant, even in the absence of histologic support for this conclusion.

Soft tissue tumors

The first occasional DNA data on soft tissue tumors originated from mixed series of different solid tumors, i.e., studies mainly focused on carcinomas. A major DNA study of soft tissue tumors was not reported until 1987 (Kreicbergs et al. 1987). In a series of 82 lesions only one (fibrosarcoma) failed to yield adequate cell material for FCM. All 23 benign tumors were diploid. In the malignant group aneuploidy was found in one of 12 grade II tumors, 21 of 29 grade III, and 15 of 17 grade IV. Among 26 malignant fibrous histiocytomas, all of high-grade (III-IV), there were only four diploid lesions; three were grade III and one was grade IV. Cell-cycle analysis, feasible in 51 cases, disclosed that the diploid tumors had a low proportion of S- and G2- + M-phase cells and most aneuploid tumors a high proportion, reflecting a relationship between ploidy level and proliferative activity. Hence, cell-cycle analysis of soft tissue tumors does not seem to provide information beyond that obtained by ploidy determination. A close relationship between histologic grade and ploidy level was also reported by Xiang et al. (1987) based on a series of 24 soft tissue sarcomas; all 16 malignant fibrous histiocytomas analyzed were histologically high-grade and aneuploid.

The most extensive DNA study on soft tissue tumors, including 82 benign and 64 malignant lesions, was reported by Matsuno et al. in 1988. According to FCM all 82 benign lesions were diploid, whereas 34 of the 64 malignant were aneuploid. Although there was a relationship between grade and ploidy, the authors considered the 80 percent rate of accuracy for the FCM analysis unacceptable for clinical use. As to cell cycle analysis, the malignant tumors showed a higher percentage of replicating cells than did the benign lesions. Notably, cellular desmoids, benign but aggressively growing, were found to have the highest percentage of cycling cells among the benign lesions. In a prognostic part of the study, based on 63 sarcomas and 30 desmoids, the authors introduced and tested the value of different FCM algorithms. None of the DNA algorithms correlated with the rate of local recurrence or distant metastasis as opposed to histologic assessment. Unfortunately, no data were given on pertinent patient and tumor features, nor on treatment. The authors concluded that there is a relationship (80 percent) between histologic grade and certain DNA algorithms in soft tissue tumors, but not sufficiently to warrant the clinical use of flow cytometry. Arguably, it is the lack of 100 percent compliance between histology and ploidy, which is of interest. Thus, the crucial issue is to establish the predictive value of ploidy level in noncompliant cases.

In a recent prospective FCM study of soft tissue sarcoma, the prognostic significance of DNA content and different clinicopathologic features was investigated by multivariate analysis (Bauer et al. 1989, unpublished data). The series included 83 patients treated by surgery. According to FCM, 32 lesions were diploid and 51 aneuploid. The 3-year, metastases-free survival rate for the whole series of 83 patients was 0.59. For patients with diploid tumors, the survival rate was 0.81 versus 0.41 for those with aneuploid tumors. Multivariate analysis identified three independent risk factors for metastasis: tumor size $\geq$ 8 cm, extracompartmental tumor location, and aneuploidy, the latter being the strongest risk factor. Unexpectedly, histologic grade was not found to be an independent risk factor for metastases. This could mainly be attributed to a high correlation between grade and ploidy, the latter, however, being the stronger predictor. This study, in contrast to the report of Matsuno et al. (1988), indicates that determination of ploidy level in soft tissue sarcoma is of predictive value.

The collective DNA data on soft tissue tumors suggest that there is a relationship between histology and ploidy in the sense that benign lesions are diploid and that among malignant lesions the incidence of aneuploidy increases with histologic grade. Whether DNA cytometry offers more information than histologic grading has yet to be established. It must be emphasized that DNA analysis cannot replace histologic assessment. Instead, it should be regarded as an objective adjunct, which, in general, supports, but occasionally questions histologic evaluation.

Provided quantitative DNA analysis is a clinically relevant means of characterizing soft tissue tumors, a combined diagnostic approach based on cytologic evaluation and DNA cytometry of fine-needle aspirates may reduce the need for open biopsy. In a study from 1987, Åkerman et al. (1987) reported that the cytologic preoperative samples from 23 of

25 soft tissue tumors were morphologically conclusive with regard to benignity and malignancy. Sufficient cell material for flow DNA analysis was obtained in 22 of the 25 tumors; the three failures were two benign tumors and one sarcoma. The only benign tumor analyzed was diploid. As to the malignant tumors, 10 were diploid and 11 were aneuploid. However, analysis of the corresponding surgical specimens revealed that in fact 16 were aneuploid. This discrepancy may explained by sampling errors at aspiration. Thus, the cells for FCM were those remaining in the syringe after ordinary smearing for cytodiagnosis. The authors, therefore, concluded that one or two additional aspirates from which all the material is flushed out for flow DNA analysis should be performed.

In a recent similar study of five benign and 21 malignant soft tissue tumors, only a desmoid failed to yield sufficient cell material for FCM (Kreicbergs 1989). Multiple fine-needle aspirates were obtained from the remaining 25 tumors. Out of a total of 113 aspirates as many as 112 provided conclusive DNA histograms. The four benign lesions were all diploid, whereas the malignant series included seven diploid, five tetraploid, and nine aneuploid lesions. A comparison with results obtained by analysis of corresponding surgical specimens disclosed complete agreement in terms of diploidy, tetraploidy and aneuploidy in all the cases except 1. Thus, in one sarcoma the aspirates were aneuploid, whereas the surgical specimen was diploid. Evidently, the surgical specimen was not representative and the tumor, hence, classified as aneuploid according to the aspirates.

These two studies show that adequate cell material can be obtained by fine-needle aspiration from soft tissue tumors to permit both cytologic and FCM analysis. It may prove that sufficient diagnostic information (benign vs. malignant, low- vs. high-grade) for correct therapeutical decisions can be obtained by the described approach, obviating the need for open biopsy.

Summary

In conclusion, all the benign soft tissue tumors analyzed so far have been been diploid, whereas the malignant entities include both diploid or aneuploid variants. Further, there is a relationship between ploidy level and histologic malignancy grade. The main value of DNA analysis seems to be that it provides objective support for histologic diagnosis as to benignity and malignancy grade. Occasionally, a discrepancy between ploidy and grade is encountered. Apart from the fact that aneuploidy precludes benignity, it may prove that ploidy level in malignancy is a better predictor of clinical course than histologic grade. However, this can only be established by analyzing each malignant entity separately, because the validity of DNA cytometry probably varies with histogenetic tumor type.

Future

At present, the experience of DNA cytometry in musculoskeletal neoplasia is too limited to permit safe conclusions about the clinical utility. Although the approach seems promising, contradictory results prevail, which to some extent may be explained by differences in methodology and histogram interpretation. Therefore, it is desirable that consensus is reached about appropriate technique and terminology. It would be advantageous if investigators using FCM also had access to MSP. The latter is particularly helpful in establishing whether FCM data displaying tumor diploidy or tetraploidy are valid, i.e., based on analysis of representative tissue.

Given these conditions, it still remains to establish for each tumor type the prognostic significance of different ploidy levels (diploidy, near diploidy, tetraploidy, aneuploidy, multiple stemlines), because the clinical relevance of these may vary between entities. Similar aspects apply to different cell cycle findings. It may prove that the percentage of S- and/or G2- +M-phase cells in some tumor entities is prognostically relevant, even more so than ploidy level. Finally, DNA data should be evaluated in relation to pertinent clinicopathologic features, preferably, by multivariate analysis.

A major concern in tumor DNA cytometry is the inability to discriminate between benign and malignant lesions, unless aneuploidy is demonstrated. Although diploidy is a typical feature of benign tumors, it is also common in low-grade tumors. Still more important, diploidy, although carrying a low probability of high-grade malignancy, does not preclude it.

In the 1980s, considerable interest has focused on assessing by FCM simultaneously with DNA content, other tumor cell features, such as nuclear size, RNA content, total protein, bromodeoxyuridine uptake, surface-membrane antigens and receptors, monoclonal antibodies, etc. Evidently, these assessments require not only differential staining of the substances, but also more advanced optical systems.

The attempts of further exploiting the potential of FCM, i.e., by multiparameter analysis have been successful, although the clinical utility remains to be established. Presumably, the new techniques will provide more information on pertinent tumor features than merely the determination of peak DNA values and percentage of proliferating cells.

In the future, molecular biology can be expected to provide oncogene characteristics of tumors, which reflect growth pattern (infiltrative vs. noninfiltrative), proliferative rate, metastatic propensity, and the like. At present, cytogenetic analysis seems to be the most informative approach in further characterizing musculoskeletal tumors beyond quantitative DNA analysis.

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