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MEETING REPORT

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Cell proliferation assessment in oncology

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Abstract A review of the current knowledge on cell cycle control and the techniques used to assess proliferation of normal and neoplastic cells was the focus of a workshop in Regensburg, Germany, held under the joint auspices of the Graduiertenkolleg: Therapieforschung Onkologie and the Committee on AgNOR Quantification. An overview of the recently discovered group of cyclins and their specific kinases, and of other proliferation-associated antigens, such as Ki67, PCNA and topoiseromase II alpha, was given. The topics continued with a reappraisal of modern imaging and flow-cytometric techniques. An update of the relation of AgNORs to cellular proliferation and differentiation was the link to presentations on clinical data, problems and strategies for standardization, as well as guidelines to establish the prognostic value of marker molecules. These lectures were supported by posters. Bringing together researchers from life sciences, technically oriented workers, pathologists, and clinicians resulted in a lively and constructive disussion, which is briefly summarized in the Concluding remarks.

Key words Cell proliferation · Proliferation markers · Oncology

How do cells cycle?

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In the life of cells, there are three optional pathways: cells may continuously proliferate, stay alive without further divisions, or die by apoptosis. The decision as to whether a proliferating cell is to proceed through the cycle is taken at two cardinal points, also referred to as checkpoints: the commitment to DNA replication at a point termed START in yeast or "restriction point" in higher eukaryotes, and the commitment to mitotic division at the end of G2. Throughout G1 phase, growth factors may influence the fate of cells by binding to specific surface receptors, which in turn activate a signalling cascade that regulates the transcription of both immediate and delayed early response genes (for review see Fantl et al., *Annu Rev Biochem* 62:453, 1993). Transcription of these genes will result in either differentiation or proliferation, the latter being promoted by convergence of receptor-mediated signals on to a "clockwork" mechanism that ensures an orderly progression through the cell cycle. Once cells have entered S phase, they become refractory to growth-factor-induced stimuli, the subsequent cell cycle events being governed by an intrinsic programme regulating the progression through mitosis.

Heteroprotein dimers consisting of a protein kinase catalytic subunit and a cyclin as a regulatory protein constitute the basic clockwork of cell cycle progression (reviewed by Norbury and Nurse, *Annu Rev Biochem*

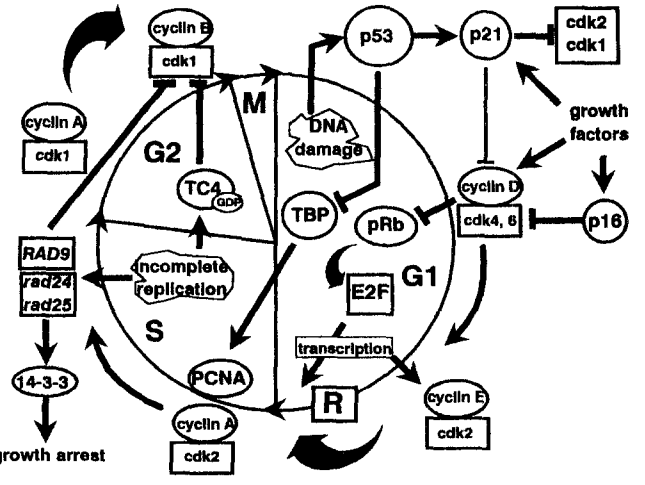

Fig. 1 Gross schematic representation of the regulatory events in the cell cycle (R=restriction point)

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62:441, 1992). In *S. cerevisiae,* CDC28 is the sole cyclin-dependent kinase (cdk) required, functioning both at the G1/S and at the G2/M transition by associating with the G1 cyclins puc1 or CLN1-3, and the G2 cyclins cdc13 or CLB1-6 (for review see Nasmyth, *Curr Opin Cell Biol* 5:166, 1993). In most higher eukaryotes, however, distinct cdks promote entry into S and M phase (van den Heuvel and Harlow, *Science* 262:2050, 1993). Their levels remain practically invariant during the cell cycle, whereas cyclins accumulate and vanish in a periodical manner by ubiquitin-mediated proteolytic degradation of type A and B cyclins and transcriptional control of cyclins D and E. The sequential association of cyclins and their dependent kinases determines the position of the cell in the cycle: growth-factor induced D-type cyclins, complexing with cdk4 and cdk6, function in early and mid-G1 phase; S-phase entry is triggered by cdk2 and cyclin E, the latter being replaced by cyclin A to regulate S-phase progression (reviewed by Sherr, *Cell* 73:1059, 1993, *Cell* 79:551, 1994). Subsequently, cyclin A associates with $p34^{cdc2/CDC28}$ (cdk1) and may play a part in the activation of the cyclin B-cdk1 complex responsible for the onset of mitosis (reviewed by Dunphy, *Trend Cell Biol* 4:202, 1994).

The cell cycle proceeds by way of well-defined successive steps, each of which must be completed before the beginning of the next. This duly progression is warranted by feedback mechanisms at so-called checkpoints. As an example, incomplete protein synthesis causes fibroblasts to accumulate in G1 (Rossow et al., *Proc Natl Acad Sci* 76:4446, 1979). One possible mechanism functioning at this point of the cell cycle was recently discovered by Baroni et al. (*Nature* 371:339, 1994): stimulation of the Ras/cAMP signaling pathway, dependent on the critical rate of protein synthesis, brakes the transit through START by repression of CLN1 and CLN2. Mitotic entry is dependent on the completion of DNA synthesis, which appears to be controlled by the products of the *RAD9* (reviewed by Hartwell and Weinert, *Science* 246:629, 1989) and RCC1 (Nishitani, *EMBO J* 10:1555, 1991) as well as the rad24 and rad25 genes. Mutants defective in these genes enter M phase prematurely, which results in a mitotic catastrophe phenotye that rapidly leads to cell death. rad24 and rad25 encode highly preserved 14-3-3 protein (reviewed by Morrison, *Science* 266:56, 1994) homologues that are claimed to link the G2 checkpoint control to the apparatus of cell cycle regulation (Ford et al., *Science* 265:533, 1994). Yet another signalling pathway of the G2 checkpoint is provided by the ras-related G protein TC4 (Bischoff and Postingl, *Proc Natl Acad Sci* 88:10 830, 1991), which, in its GDP-bound form, inhibits $p34^{cdc2}$ by phosphorylation of tyrosine and threonine residues. Completion of DNA synthesis then mediates the expression of RCC1, shifting TC4 to the GTP-bound form that releases the brake (Kornbluth et al., *J Cell Biol* 125:705, 1994).

Other proteins also have the properties of checkpoint controls. The protein product of the retinoblastoma susceptibility gene Rb plays a crucial role in the negative regulation of the cell cycle (for review see Nevins, *Science* 258:424, 1992).

Loss of control mechanisms at either checkpoint will result in gene amplification and genomic instability, a hallmark of malignant transformation (reviewed by Hartwell and Weinert, *Science* 246:629, 1992). One important reason for checkpoint failure is the abrogation of tumour suppressors. Rb is known to be inactivated by viral oncogenes, such as SV40, E1A, E6 and E7, which bind to its hypophosphorylated form (see Nevins, *Science* 258:424, 1992). The p53 gene is prone to spontaneously occurring missense and nonsense mutations, which preferentially affect the DNA-binding core of its protein product (Cho et al., *Science* 265:346, 1994), generating loss-of-function mutants that are found in a wide variety of cancers (see Levine et al., *Br J Cancer* 69:409, 1994).

Deregulated cell growth is in part reflected by the expression of characteristic proliferation-associated proteins, some of which are presently detectable by immunohistochemical methods (Gerdes et al., *J Immunol* 133:1710, 1984; Sampson et al., *J Pathol (Lond)* 168:179, 1992; Kreipe et al., *Am J Pathol* 142:1689, 1993).

We have generated several monoclonal antibodies (Mabs) to a set of DNA-binding proliferation-associated antigens (Rudolph et al., in preparation), such as that defined by the antibody Ki-67, whose function is still unknown despite the cloning of its cDNA (Schlüter et al., *J Cell Biol* 123:513, 1993). Currently, we are analysing other cell-cycle-related nuclear proteins differentially expressed during the different phases of the cell cycle, or visualizing the transit through the restriction point, in order to gain a deeper insight into the mechanisms involved in cell cycle regulation.

Immunocytochemical assessment of cell proliferation

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There is little doubt that proliferation is an important feature in characterizing the malignant phenotype and biological behaviour of neoplasms. However, it appears to be rather the exception than the rule for the proliferative activity to be used as a prognostic indicator in clinical decision making. Counting mitotic figures is the traditional morphological approach to assessing cell proliferation. Several careful studies have provided evidence for the prognostic impact of mitotic counts (Baak, *Hum Pathol* 21:683, 1990). Criticism of this method concerns problems in standardization and reproducibility.

The immunohistochemical method requires that the antigen recognized is expressed exclusively in cycling cells or that there is a cell-cycle-induced increase in antigen expression. There are a number of cell-cycle-related structures that fulfil these requirements, and against

those antibodies are available for immunohistochemistry. These antigens can be grouped into two categories. One comprises enzymes and cofactors that are involved in DNA synthesis during the S-phase of the cell cycle. The second group represents proteins of unknown function that are detectable in proliferating cells. Markers belonging to the first group are DNA polymerase alpha, topoisomerase II alpha and proliferating cell nuclear antigen (PCNA). The most famous example in the second group is Ki-67, others are p105 (Clevenger et al., *Exp Cell Res* 151:194, 1984) and JC1 (Garrido et al., *J Clin Pathol* 45:860, 1992).

It seems reasonable to expect that enzymes involved in DNA synthesis are suitable for immunohistochemical detection of proliferating cells. The most frequently used marker in this group is proliferating cell nuclear antigen (PCNA) that represents a 36-kDa non-histone nuclear protein, which functions as an auxiliary protein for DNA polymerase delta (Bravo et al., *Nature* 326:515, 1987). Monoclonal antibodies suitable for the detection of PCNA in paraffin-embedded tissues have been developed by immunizing mice with recombinant PCNA protein (Waseem et al., *J Cell Science* 96:121–129, 1990). In normal tissues the PCNA monoclonal antibody PC10 yields a staining pattern that is consistent with the recognition of the proliferative compartment (Hall et al., *J Pathol (Lond)* 162:285, 1990). In malignant tumours conflicting results have been published concerning the value of PCNA in the determination of the growth fraction. Some studies found a good correlation with other markers of cell proliferation (Dervan et al., *Am J Clin Pathol* 97: Suppl 21, 1992; Kamel et al., *J Pathol (Lond)* 138:1471, 1991). Others did not find any correlation at all. (Leonardi et al., *J Clin Pathol* 45:416, 1992; Visakorpi et al., *J Pathol (Lond)* 168:7, 1992). The reactivity pattern of PCNA staining is also influenced by the fixation procedure with methanol fixation leading to the labelling of cells in S-phase, whereas other fixation procedures allow recognition of all actively cycling cells (Landberg et al., *Cancer Res* 51:4570, 1991). Formaldehyde-based fixatives have been shown to lead to a weak to non-existent staining, resulting in a much lower percentage of labelled cells (Rowlands et al., *J Pathol (Lond)* 165:356, 1990).

Besides these technical aspects there are biological aspects that complicate the interpretation of PCNA staining. Thus, there is evidence for deregulated overexpression in some types of malignant tumours (Hall et al., *J Pathol (Lond)* 162:285, 1990), and the long half-life of the protein leads to labelling of cells that have already left the cell cycle (Scott et al., *J Pathol (Lond)* 165:173, 1991). Furthermore, PCNA may be induced in normal non-cycling cells adjacent to tumours (Hall et al., *J Pathol (Lond)* 162:285, 1990). In bone marrow cells only proerythroblasts, and no later proliferating cells of this lineage, were stained, and the reasons for this were unclear (Wilkins et al., *J Pathol (Lond)* 166:45, 1992). It was stated earlier that PCNA staining may occur in non S-phase cells engaged in nucleotide excision DNA repair (Celis et al., FEBS 209:277, 1986). Ki-S1, which recognizes topoisomerase II alpha, showed a good correlation with the S-phase fraction in mammary tumours (Camplejohn et al., *Br J Cancer* 67:657, 1993), whereas this was not the case with PCNA (D. Barnes, personal communication). Topoisomerase II alpha and DNA methyltransferase may be expressed at low levels in non-cycling cells and the level of expression is increased by many times when the cells enter the cell cycle. Because staining intensities can hardly be estimated by light microscopical evaluation, it is necessary to use antibody dilutions that allow the discrimination of heavily stained cells. Whereas image analysis enables the determination of different staining intensities, this is only possible in light microscopic analysis when different dilutions of antibodies are used. The relevance of antibody concentration for the immunohistological quantification of cell proliferation associated antigens has recently been stressed (McCormick et al., *Histopathology* 22:543, 1993).

In the second group comprising cell-cycle-associated proteins with unknown function, the most important is the Ki-67 antigen. The Ki-67 antigen is 345 kDa and 395 kDa in size and the corresponding gene has recently been cloned (Schlüter et al., *J Cell Biol* 123:513–522, 1993). The deduced aminoacid sequence did not reveal homology to known cell cycle proteins and its function remains elusive. Despite its unknown function the Ki-67 protein has proven to be a useful marker for cycling cells. Compared with PCNA, it yielded a far better correlation with the ^{3}H-thymidine labelling index of tumour xenografts in mice (Scott et al., *J Pathol (Lond)* 165:173–178, 1991). Although the Ki-67 index in the xenografts produced a constant overestimate of the growth fraction it followed the same trend as the classical method of mitosis labelling by incorporation of tritium-labelled thymidine (Scott et al., *J Pathol (Lond)* 165:173–178, 1991). In several tumours, in particular malignant lymphomas and mammary carcinoma, several studies have shown the Ki-67 antigen to be a significant prognostic indicator (Brown et al., *Histopathology* 17:489, 1990). Recently monoclonal antibodies have been introduced that recognize the Ki-67 antigen in paraffin-embedded tissues after microwave antigen retrieval (MIB 1–3, Key et al., *Lab Invest* 68:629, 1993; Ki-S5, Kreipe et al., *Am J Pathol* 142:1689, 1993). In our hands Ki-67 antigen staining by Ki-S5 as well as MIB 1 has proven to be a reliable instrument to assess the proliferative activity of tumours in routine surgical pathology.

If immunohistochemical evaluation of proliferative activity in tumour cells is considered one should be aware of the fact that they may be heterogeneous in terms of cell cycle length. The proliferation rate of a cell population may depend on the latter parameter as well as on the growth fraction. Immunohistochemical proliferation markers like Ki-67 can provide information only on the latter. Despite these potential shortcommings the immunohistochemical detection of proliferation antigens such as Ki-67 can give significant prognostic information. With standardized staining and evaluation protocols

it should be possible in the future to exploit reliable markers of cell proliferation as a source for additional prognostic information in the grading of neoplasms.

Cell biological basis of AgNOR staining

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One of the earliest events to occur following stimulation of cell growth is a change in the rate of ribosome precursor synthesis, suggesting a close relationship between the rate of cell proliferation and ribosome biogenesis. It is therefore of general interest to possess markers of the level of ribosome biogenesis correlated with cell proliferation. The best example of this correlation is provided by a set of proteins associated with the ribosomal genes whose amount is directly related to ribosomal gene transcription and/or cell duplication. These proteins, called AgNOR proteins, are defined as markers of "active" ribosomal genes. They correspond to a set of proteins specifically located in the NORs that are identified by their ability to reduce silver under acidic conditions in which other cellular proteins remain unstained (Howell et al., *Experientia* 36:1014, 1980; Goodpasture and Bloom, *Chromosoma* 53:37, 1975).

The interest in this set of proteins started when it was demonstrated that transcription of the ribosomal genes depends on the presence of AgNOR proteins (Miller et al., *PNAS* 73:4531, 1976). On metaphase chromosomes, a positive AgNOR staining is the marker of the ribosomal gene transcription taking place during interphase. To define the role of the AgNOR proteins, we investigated their localization during cell cycle, and we developed a procedure to identify and quantify single AgNOR proteins in different cells.

Localization of AgNOR proteins

The specific affinity of the AgNOR proteins for silver allowed their localization by electron microscopy in situ in nucleoli and on molecular spreadings of ribosomal transcription units. By electron microscopy, they were found in the fibrillar centres (FC) and the dense fibrillar component (DFC) of the nucleoli and excluded from the granular component (GC) in interphase nuclei (Hernandez-Verdun et al., *Chromosoma* 102:146, 1980). After molecular spreadings of nucleoli, these proteins were found exclusively on the transcribed part of the ribosomal genes and were not observed in spacer regions. The most rapidly stained proteins seemed located on the DNP axis rather than on the RNP fibrils (Angelier et al., *Chromosoma* 86:661, 1982).

The 3-dimensional (3-D) distribution of the AgNOR proteins have been investigated using confocal microscopy in reflexion mode (Robert-Fortel et al., *Chromosoma* 102:146, 1993). During interphase, AgNOR proteins form granules linked by a thin filament in the inner regions of the nucleoli whereas in the same sites the ribosomal genes distribute as intense fluorescent spots linked by weak signals. These images indicate similar 3-D distributions for ribosomal genes and AgNOR proteins in nucleoli. In metaphase chromosomes, the AgNOR proteins form a crescent-shaped structure around the axial chromatin pedicule in the secondary constriction. The ribosomal genes are observed both in axial chromatin pedicule and also lateral expansions.

Identification of AgNOR proteins

We adapted the specific detection method of the AgNOR proteins to Western blots in order to identify these proteins. We tested the most commonly used cytochemical AgNOR staining techniques (Hozak et al., *J Histochem Cytochem* 40:1089, 1992). A one-step method with colloidal developer was standardized to obtain reproducible results. The specificity of AgNOR staining is documented by the facts that only a few bands are revealed among the many proteins detected by total protein staining and that the reaction is specific for a subset of nucleolar proteins; the same bands are observed with the use of nucleolar, nuclear or total cell protein extracts. This specificity for nucleolar proteins mimics the AgNOR staining of cells in situ.

Using AgNOR staining on Western blot proteins we found that purified nucleolin is specifically revealed by AgNOR staining. Using different nucleolin fragments generated by CNBr cleavage and by overexpression in *Escherichia coli,* we demonstrated that the aminoterminal domain of nucleolin, and not the carboxy-part of the protein, is involved in silver staining. Moreover, as the pattern of staining does not vary using casein kinase II- and cdc2-phosphorylated nucleolin or dephosphorylated nucleolin, we concluded that the reduction of the silver ions is not linked to the phosphorylation state of the molecule. We propose that the concentration of acidic amino acids in the amino-terminal domain of nucleolin is responsible for AgNOR staining. This hypothesis is also supported by the finding that poly L-glutamic acid peptides are silver stained. These results provide data that can be used to explain the specificity of AgNOR staining (Rousell et al., *Exp Cell Res* 203:259, 1992).

AgNOR proteins were identified at specific phases of cell cycle. During interphase, the major Ag-NOR proteins in both human and hamster cell lines are nucleolin and protein B23, and also proteins of 42, 40 and 29 kDa, that account for a small amount of the silver stain (Roussel and Hernandez-Verdun, *Exp Cell Res* 214:465, 1994). The p*I*s of these proteins was between 4.5 and 5.6. During mitosis, the major Ag-NOR proteins associated with the ribosomal genes are the largest RNA polymerase I subunit, the 135-kDa NOR protein, the UBF transcription factor and a 50-kDa protein. Thus the major Ag-

NOR proteins in nucleoli during interphase are not the same as those associated with the ribosomal genes during mitosis. We conclude that the prognostic test for human cancer cell proliferation is largely based on the amount of the nucleolar proteins, nucleolin and protein B23, that are not directly involved in ribosomal gene transcription. In contrast, the evaluation of "active" NORs in karyotypes during mitosis is based on the presence of some proteins of the ribosomal gene transcription machinery.

Quantification of single AgNOR proteins

Quantification of AgNOR proteins by image analysis is currently used to evaluate the rate of proliferation of cancer cells and nucleolar activity. Our objective was to establish a procedure to quantify independently each major AgNOR protein in cell extracts. Computerized densitometry established that the specific silver staining of AgNOR proteins performed on Western blots makes it possible to quantify AgNOR proteins (Roussel et al., *J Histochem Cytochem,* in press). Using purified Ag-NOR proteins, nucleolin and protein B23, it was observed that the intensity of AgNOR staining is proportional to the amount of protein. A linear relationship exists between the intensity of AgNOR staining and amount of nucleolin in the range of 0.2–1.6 μg. Using total nuclear extracts prepared from mammalian cells, the proportionality was maintained for total AgNOR-stained proteins or for a particular protein. We also determined the levels of nuclear proteins suitable for quantitative analysis. Thus, individual AgNOR proteins can be quantified by computerized densitometry in nuclear extracts after AgNOR staining on Western blots. This procedure may be applied to establish the contribution of each AgNOR protein in general staining, to estimate the variability of each AgNOR protein in normal and pathological conditions, and to quantify each AgNOR protein contained per cell.

Current status and perspectives of image analysis: analysis of FISH-stained cells and chromosomes

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Quantitative analysis of images of cells and tissues may be used for both research and diagnostic clinical applications. Examples of such applications are the quantification of DNA ploidy and of hormone receptors, as a prognostic tool in oncology, or in the morphometric analysis of cells and tissues. In addition, analysis of cells and chromosomes stained by fluorescence in situ hybridization (FISH) has become a major application, as instrumentation and reagents have become available commercially. At present, quantitative analysis of FISH images is becoming increasingly important in research and diagnosis. This paper describes its main applications and focuses on its future impact.

In situ hybridization allows the detection of specific nucleic acid sequences in morphologically intact cells and chromosomes. At present, FISH has reached a high detection sensitivity (1 kB or less), a high DNA resolution (1 kB: defined as the smallest distance in kB between two DNA targets that can be resolved microscopically), and a high multiplicity (up to 12) (defined as the number of different probes that can be identified simultaneously (Dauwerse et al., *Hum Mol Genet* 1:593, 1992; Nederlof et al., *Cytometry* 11:126, 1990). In addition, the introduction of sensitive charge-coupled device (CCD) cameras for the recording and analysis of FISH stained cells and chromosomes has significantly contributed to the current status of FISH (Arndt-Jovin et al., *Sciences* 230:247, 1985; Aikens, In: Optical microscopy for biology, ed. by Herman and Jacobsen, Wiley-Liss, New York 1990). These are important applications of analysis of FISH images.

Interphase cytogenetics

The major advantage of interphase cytogenetics (Cremer et al., *Hum Genet* 80:235, 1988) is that it does not require cell culture to generate metaphase chromosomes, and provides information about each cell in the population. The method is relatively simple, fast and can provide clinically useful information (Hopman et al., *Cancer Res* 51:644, 1991). In addition probes that hybridize with defined chromosome domains are commercially available. Numerical chromosomal aberrations can thus be studied. However, the counting of spots in interphase cells and the statistical evaluation of the results deserves attention especially in cases where the frequency of the studied mosaicism is low, for instance in minimal residual disease (Kibbelaar et al., *Cytometry* 14:716, 1993). Under such circumstances the FISH results of many nuclei have to be evaluated, which is practically only possible by computerized microscopy. Nuclei can be detected on the basis of the counter stain used and within each detected nucleus the number of FISH spots is determined. Artefacts such as dirt, overlapping nuclei and border objects are thereby eliminated. The results are presented to the operator in two ways: as a gallery containing the nuclear images and the proposed count, and simultaneously as a histogram. Mistakes in these galleries are rapidly corrected by the operator; subsequently the histogram is automatically adapted accordingly.

Comparative genomic hybridization

Recently, a new technique has been proposed to analyze DNA isolated from basically any tissue for the presence

gains and losses of genes (Kallioniemi et al., *Sciences* 258:818, 1992). This technique, called comparative genomic hybridization (CGH) generates a copy number karyogram. The principle of the method is that DNA isolated from malignant cells (labelled green) is mixed with normal DNA (labelled red) and hybridized to normal human interphase chromosomes. Repetitive sequences are thereby blocked by using unlabelled Cot-1 DNA. Individual chromosomes are identified on the basis of counterstaining with DAPI, which results in a banding pattern. The green-red ratio image of each identified chromosome is subsequently recorded by digital imaging microscopy. In the case of normal DNA homogeneously orange-stained chromosomes are observed; areas that fluoresce predominantly red or green indicate gene loss or amplification, respectively. Unlike other FISH methods to detect amplification or deletion, no a priori knowledge or a gene-specific probe is required. In principle, the method is suitable for study of any unbalanced genetic material; obviously, balanced translocations will remain undetected. Potential applications for CGH are many: it is reasonable, however, to anticipate that genes may be detected that play a role in tumorigenesis or that are related to metastatic potential.

Radiation-induced genetic abnormalities

Quantitative assessment of the effects of radiation is important for the detection of occupational and environmental exposure, for the evaluation and guidance of medical treatment in cases of accidental exposure, or for the measurement of radiosensitivity of tumours and normal tissue to optimize cancer therapy. It is known that the scoring of stable aberrations (translocations) is a good estimator when the exposure is chronic or when the analysis is carried out at long times after irradiation. Detection of translocations can be accomplished by using whole chromosome painting probes in combination with a counter stain for total chromosomal DNA. Translocations can then be scored by counting the number of chromosomes containing parts of the painting probes in relation to the original number of painted chromosomes.

A system has been developed for the automated detection and quantitation of such translocations.

Gene mapping

In the process of positional cloning of disease genes, DNA mapping of the genome region of interest is essential. It has been shown that FISH staining of genes on chromosomes and in interphase nuclei in combination with simple measurements of the distance between the various hybridization spots provides an accurate tool to determine the genomic distance. The resolution of such mapping techniques depends strongly on the compactness of the studied DNA, and is approximately 3 Mb on condensed metaphase chromosomes and about 100 kb in interphase nuclei. As an alternative to interphase nuclei a nuclear extraction technique has been proposed that, by detergent and high-salt extractions and a DNA relaxation treatment, results in chromatin that is decondensed to the level of the Watson-Crick DNA double helix. In situ hybridization to such DNA-halo preparations may contribute to DNA mapping efforts at relatively short range (1–200 kb) as well as to the characterization of breakpoints, microdeletions and small duplications (Wiegant et al., *Hum Mol Genet* 1:587, 1992).

Principles of proliferation analysis by flow cytometry

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Research on growth factors, oncogenes and intracellular activation pathways like cyclins/cycline-dependent kinases and phosphatases and transcription factors has immensely improved our understanding of the regulation of cell proliferation. Eukaryotic cell proliferation is regulated via different signals at various physiological and biochemical levels during the cell cycle, which is basically dissected into following compartments: growth and physiological differentiation functions (G1 phase), identical reduplication of the eucaryotic genome (S, G2M phase), or performance of specialized physiological or memory functions (GO phase).

The methodological improvements of measuring the cellular response to mitogenic signals has lagged behind the major cell biological advances. Historically, by light microscopy, mitosis was recognized as a distinct compartment of the cycle, and when radiolabelled nucleotide precursors were introduced into DNA the DNA-synthesis phase could be differentiated. However, the GO, G1 and G2 phases cannot be observed and quantified by these techniques. Even the introduction of nonradioactive assays like the MTT assay did not overcome the limitation of resolving the complexity of the cell proliferative processes in vitro. Most of these techniques, including various refinements, are either time-consuming, radioactive, technically complex or beset by the impossibility of dissecting the heterogeneity of cell population kinetics in both normal diploid and permanent cell systems in vitro or in vivo.

Some drawbacks of the radioactive labelling techniques have been overcome by the introduction of flow-cytometric assessment of DNA content as a measure of cell activation and cell cycle progression (Gray and Darzynkiewicz, Techniques in cell cycle analysis, Human Press, Clifton, NJ, 1987). Application of compartment-specific cell cycle blockers, such as Colcemid, yielded some information on the kinetics of cells in the cell cycle of different compartments (stathmokinetics). When DNA and RNA measurements were combined or structural DNA-chromatin changes, were used, however flow cytometry achieved an unprecedented resolution of the kinetic events associated with cell activation.

The recent application of flow cytometry with immunocytochemistry has added yet another technical dimension. Antibodies against activation- and replication-specific nuclear antigens (Ki67, PCNA, KiS1) enable quantification of proliferating cells in vitro as well as in vivo (Kreipe et al., *Am J Pathol* 142:3, 1993). In a multiparameter fashion the presence of these antigens is analysed as a function of DNA content to obtain information about cell-cycle-related expression.

To acquire complex flow cytometric information about the kinetics of proliferation, however, DNA labelling is required. In the majority of applications the nucleoside BrdU is added in vitro or in vivo and incorporated during semiconservative replication into DNA. Using monoclonal antibodies against pulse-labelled, BrdU-substituted DNA and conventional DNA content analysis, replicating and non-replicating cells are clearly distinguished and cell cycle traverse rates can be quantitated. This immunocytochemical technique is quite sensitive, enabeling in vivo labelling of replicating cells and subsequent flow cytometric analysis. In some circumstances, additional cell subpopulation analysis might be possible. One limition by this BrdU immunocytochemical technique, however, is that the G1 product of mitotic, BrdU-labelled cells is indistinguishable from the G1 or G0 cells that have never entered S phase (Omerod and Kubbies, *Cytometry* 13:678, 1992).

The most complex cell cycle analysis is possible with the continuous BrdU-labelling BrdU/Hoechst quenching technique (in: Radbruch (ed) *Flow cytometry and sorting*, Springer, Berlin, p 75, 1992; Giese et al., *J Cell Physiol* 161:209, 1994). BrdU incorporation is detected by the reduction of the fluorescence intensity of the Hoechst dye. Optimizing the BrdU-Hoechst staining technique, high resolution histograms were obtained using either one- or two-dimensional flow cytometric techniques. In synchronous cell populations at least three different cell cycles can be differentiated and quantified. Additionally, a wealth of kinetic data about each cell cycle and its compartments is obtained by taking histograms at multiple time points after growth stimulation. With these high-resolution techniques, the heterogeneity of the cell population kinetics could be resolved for the first time, giving insights into problems like early- and late-replicating cells after growth stimulation, cycling and non-cycling cells, transient vs complete cell cycle compartment arrest and cell cycle and compartment durations. When the BrdU/Hoechst flow cytometric quenching technique is applied to asynchronous cell populations for the first time, even the G1 and G2 phases can be displayed stretched as a function of time, like the S phase compartment. In addition, immunocytochemistry of intracellular or surface marker is possible in a multiparameter analysis with BrdU-Hoechst staining. Finally, the BrdU-Hoechst analysis of viable cells with PI staining gives insight into the cell death kinetics of cell populations either via necrosis and/or apoptosis.

Depending on the scientific problem, biological system (e.g. in vivo or in vitro) and/or laboratory equipment (UV or visible light excitation) one of several flow cytometric proliferation techniques might be selected. However, except for high thruput systems in screening laboratories, there is no doubt that flow cytometry offers the most advanced technique and highest resolution of cell activation/competence and cell cycle progression analysis of cell proliferation.

Principles of laser scanning microscopy

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Introduction

Laser scanning microscopes are more and more used in various fields, particularly in biological and biomedical research. The main reason for this attention is the property of optical sectioning of specimens without the need to cut the object physically. Optical sectioning is achieved by a confocal arrangement of the optics which will be explained in detail below. As many commercial confocal microscopes use only laser illumination (Biorad, Leica, Lasertec, Sarastro, Zeiss) there is a popular notion that confocal microscopes must use laser illumination. However, confocal microscopy does not necessarily require laser light (In: Pawley (ed), *Handbook of biological confocal microscopy:* 69, Plenum Press, New York, 1990). In laser-based microscopes the field is scanned point by point by the diffraction pattern of a single-mode laser beam that has been expanded to fill the illuminating lenses. The photons emitted from the object are collected by highly sensitive photodetectors (usually photomultiplier tubes), and an image is built up digitally pixel by pixel, with the brightness of each pixel proportional to the number of photons emerging from the corresponding point in the object.

The confocal principle

The basis of confocal imaging are two pinholes arranged confocally on the optical axis of the system (i.e. they are located in conjugated foci of the objective lens). The objective lens forms an image of the illumination pinhole A onto a confocal spot in the specimen, B. The light emerging from B passes back through the objective lens which forms an image of B onto the second pinhole, C, located in front of the photodetector. The points A, B and C are confocal points. The property of optical sectioning arises from the restriction of the detector area by the second pinhole C. If C was infinitesimal, exclusively photons emerging from the diffraction ellipsoid around the focal point of the objective lens would be able to pass through C onto the detector. Hence, by (horizontally) scanning the illuminating beam over the object an image of diffraction-limited thickness is built up pixel by pixel.

Now, if the object is shifted along the optical axis, planes in different depths of the object can be imaged sequentially, i.e. the object can be sectioned optically. In practice, however, the detector pinhole will not be infinitesimal, and hence the thickness of the imaged slab in the object (i.e. the optical section) will be of finite thickness. The restriction of the depth of field is the one major consequence arising from the spatial limitation of the detector.

The second consequence arising from the spatial limitation of the detector is the improvement of the resolution governed by the three-dimensional diffraction pattern of the optical components. In the case of an infinitesimal detector whose sensitivity can be expressed mathematically as a Dirac delta function the recorded image of a point object is described by the fourth power of a first-order Bessel function, while the point response of a conventional microscope is governed by the second power of the first-order Bessel function. Calculation of the resolution based on the half-width of these point responses shows that the lateral resolving power of the confocal instrument is approximately 1.4 times the resolving power of a conventional microscope. For the case of a finite sized pinhole, however, the improvement of the resolution is somewhat lower, depending on the size of the detector (Wilson et al., *J Microsc* 149:51, 1988; Wilson, In: Pawley (ed) *Handbook of biological confocal microscopy:* 113, Plenum Press New York, 1990). The axial resolving power is approximately one half of the lateral resolving power (Inoue, In: Pawley BJ (ed), *Handbook of biological confocal microscopy:* Plenum Press New York, 1990) and less sensitive to pinhole size (Brakenhoff et al., *J Microsc* 153:151, 1989).

It should be stressed that in conventional fluorescence microscopy the focused image is blurred by light emitted from object points that are out of focus. Hence the depth sectioning capacity of a conventional fluorescence microscope is close to zero despite the finite vertical resolving power.

The resolution of an optical system is strongly influenced by the coherence of the radiation used to form the image. Incoherent systems obtain an enhanced resolution compared to coherent systems operated under the same conditions (same wavelength and numerical aperture). This can be explained by the lack of interference effects in incoherent microscopy. In biological applications most of the specimens are observed in fluorescence mode, hence in spite of the (coherent) laser illumination the final image is formed by the incoherently emitted fluorescence radiation. The lateral resolving power of fluorescence confocal microscopes is therefore improved by a factor of 2 compared with conventional microscopes.

Applications of confocal laser scanning microscopy

It should be clear that one of the main advantages of confocal microscopes is the ability to analyse parts of thick objects in situ. In our own laboratory, for example, we examined the effects of ultrasound shock waves on human umbilical endothelial cells in situ. Following staining of the F-actin filaments, alterations of the endothelial cytoskeleton could be clearly observed (Seidl et al., *Ultrasound Med Biol,* in press 1994; Steinbach et al., *Eur J Ultrasound,* in press 1994). Due to the underlying tissue containing high amounts of actin this kind of analysis would have been completely impossible with a conventional microscope. Similarly, we could demonstrate damage to cells inside threedimensionally grown tumour cell spheroids (Steinbach et al., *Ultrasound Med Biol* 18:691, 1992) and inside kidney tissue (Roessler et al., *Urol Res* 21:273, 1993) using vital dyes.

Laser scanning microscopy can most successfully be employed in proliferation analysis if the intracellular distribution of proliferation related proteins is of specific importance. A well-known example is the cell-cycle-dependent distribution of the nuclear matrix-associated proliferation related antigen Ki 67 that was analysed by Verheijen et al. (*J Cell Science* 92:123, 1989). More recent work deals with the localization of protein kinase C isozymes in order to get insight into the mechanisms regulating apoptosis (Knox et al., *Exp Cell Res* 207:68, 1993) or with the cellular distribution of proliferation cell nuclear antigen, cyclin A, CDC-2, c-myc, and p53 proteins (Gazitt et al., *Cancer Res* 54:950, 1994).

Today, confocal microscopy is far from being a standard tool in clinical diagnosis, however there are some examples of its use in clinical or cytological diagnostics. An improvement of the cytological diagnosis of cervical smears by laser scanning microscopy is proposed by Boon et al. (*Acta Cytol* 37:40, 1993). The future progress of a clinical use of confocal microscopy will be heavily dependent on the development and establishment of marker molecules whose cellular distribution is of prognostic relevance.

Laser scanning microscopy and its application to NOR analysis

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Nucleolar organizer regions (NORs) were initially described as regions around which nucleoli reorganize during telophase (for review see: Howell, in: Bush et al. *The cell nucleus,* vol XI, Academic Press, New York 1980). By means of the in situ hybridization technique, rDNA genes were localized in these regions as well as within secondary constrictions of human metaphase acrocentric chromosomes and within nucleoli (active rDNA genes) or nucleoplasm (inactive rDNA genes) during interphase (Wachtler et al., *Exp Cell Res* 167:227, 1986).

Nowadays, NORs are defined as sites where active (or potentially active) rDNA genes are localized, i.e. nucleo-

lar fibrillar component during interphase (fibrillar centres and dense fibrillar component), secondary contrictions of metaphase chromosomes and specific sites during telophase.

Typically, NORs are also characterized by the continuous presence of several proteins which are necessary for rRNA transcription and/or processing such as RNA polymerase I (R.P.I), upstream binding factor (UBF), nucleolin (or C23 protein) and AgNOR proteins. By studying the precise localization of these proteins, one can hope to get highly valuable data on the volumic organization of the nucleolus or of components containing rDNA genes. These data can be very useful both in fundamental biology (to study the organization of the nucleus and of the nucleolus) and in pathology (see the example of AgNOR proteins). The current goal of our group is to study the 3D organization of the nucleolar components during interphase and mitosis of cancerous cells by investigating the precise localization of proteins of the NORs. To attain this goal we perform immunocytochemical and cytochemical labelings to identify the proteins of the NORs and we then visualize the stained components either in high-voltage electron microscopy (300 kV STEM) (Beorchia et al., *J Microsc* 170:247, 1993) or in confocal laser scan microscopy (CLSM; Ploton et al., *J Histochem Cytochem* 42:137, 1994).

One of the most important applications of CLSM is to perform optical sectioning, i.e. to give an image of objects only localized within the focal plane of the objective. This image has a very high contrast and has a resolution between the one of non-confocal microscopy and the one of electron microscopy. Typically, the resolution in CLSM is 0.15 μm in the *X-Y* plane and of 0.5 μm along the optical axis (*Z*-axis). Owing to the limited thickness of the optical section, it is necessary to investigate the whole volume of a cell through numerous optical sections (Z-series), which are then used to determine the 3D organization of the labelling. By using classic software one can rapidly obtain extended focus, transverse sections, rotation and anaglyphs, which are essential for an accurate idea of the 3D organization of the labelling. Typically, one or several fluorescent labellings may be studied alternately or simultaneously. Moreover, observation of one metallic labelling is also possible in reflected light simultaneously with one fluorescent marker.

In our recent studies, we investigated the 3D colocalization of AgNOR proteins in reflected light and of DNA specifically stained with chromomycin A3. We also investigated the 3D colocalization of immunolabelled RPI, UBF, and Ki-67 with that of DNA. Moreover, to show the numerous data obtained during these investigations better, we also developed two new software programs. The first one presents the cell studied as a transparent parallelepiped showing three faces, on which DNA and the marker appear simultaneously. The second one considers the fluorescent object as a solid reflecting the light more or less (ray-tracing method). This software also allows one to show the objects from various angles whose images were used to create an animated series. This technique has been used to examine the 3D colocalization of DNA with RPI, AgNOR proteins, UBF and Ki-67 antigen. During interphase these molecules (except Ki-67 protein) are found in spheres (RPI) or irregular dots (AgNOR) proteins and UBF), which are organized as several large and twisted necklaces. This typical structure corresponds to the labelling of fibrillar centres (RPI), of fibrillar centres and loops of dense fibrillar components (DFC) (AgNOR proteins) or of DFC only (UBF). Each necklace can be interpreted as one decondensed NOR (initially one cluster of rDNA genes found at the level of one metaphasic secondary constriction).

After the desorganization of the nucleolus during early mitosis, the metaphase labeling of RPI, AgNOR proteins and UBF is typically found as doublets of spheres and as crescent-shaped structures both integrated within chromosomes. Our 3D study clearly showed that these NORs are centrally located within the set of chromosomes, indicating a non-random organization of chromosomes during mitosis. During anaphase, the labelling of DNA demonstrates a typical hollow half-sphere structure of the two sets of chromosomes in which NORs are symmetrically organized in 3D. Finally, the reorganization of the nucleoli at the beginning of interphase is characterized with the fusion of proteic material (containing Ki-67 antigen) around the NORs.

In conclusion, our study indicates that CLSM and 3D visualization of data are very useful tools for investigation of the 3D organization and modification of the NORs during interphase and mitosis.

This approach clearly indicated that nucleolar proteins are not localized at random during interphase and mitosis, and this appears as another example of the strict organization of the genome. Finally, we can hypothesize that such methodologies will be very useful in a near future to study the 3D organization of the nucleus and of the nucleolus within cancerous cells treated with anticancerous drugs.

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Rationale for the use of interphase AgNOR parameters in tumour pathology

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During the past few years, after the pioneering work by Ploton et al. (*Histochemistry* 18:5, 1986) showing that prostatic cancer cells have a greater quantity of interphase AgNORs than corresponding hyperplastic cells, a lot of studies have been carried out in order to define the importance of interphase AgNOR quantification in tu-

mour pathology (reviewed in: Crocker, *Curr Top Pathol* 82:91, 1990; Derenzini and Ploton, *Int Rev Exp Pathol* 32:150, 1991; Derenzini and Trerè, *Virchows Arch [B]* 61:1, 1991).

Interphase NORs represent structural-functional units for pre-ribosomal RNA synthesis. All the components known to be necessary for ribosomal transcription are distributed within the confines of interphase NORs. In addition to ribosomal DNA, the two main enzymes controlling transcription, RNA polymerase I and topoisomerase I are located in the interphase NORs together with the upstream binding factor (necessary for the activation or rRNA transcription), fibrillarin (required for pre-rRNA processing) and a group of acidic proteins, yet not completely defined, which are selectively stained by silver methods (Howell, *Selective staining of nucleolus organizer regions [NORs].* In: Busch and Rothblum (eds) *The cell nucleus,* Academic Press, New York, 1982). These proteins that are responsible for the stainability of NORs by silver are called AgNOR proteins, and the NORs identified in this way, AgNORs. Transcription of ribosomal genes is dependent on the presence of the AgNOR proteins. The two main AgNOR proteins are nucleolin (also called protein C 23) and protein B 23. Nucleolin induces ribosomal gene decondensation by binding to the histone H1; it is also associated with nascent pre-RNA. Protein B23 plays a role in the latter stages of rRNA processing being associated with mature RNP nucleolar components (Olson, *The role of proteins in nucleolar structre and function,* in: Straus and Wilson (eds) *The eucaryotic nucleolus: molecular biochemistry and marcomolecular assemblies,* vol 2, Telford Press, Caldwell NJ, 1990).

According to their function in ribosomal biogenesis, it is not surprising that the number of interphase AgNORs is highly variable, depending on the metabolic activity of the cell. In resting cells stimulated to proliferate it has been shown that the number of interphase AgNOR proteins measured by morphometric analysis in cytohistological samples increases in parallel with the increase in rRNA synthesis, reaching their highest value at the end of the S phase (Derenzini and Trerè, *Virchows Arch [B]* 61:1, 1991). Very recently using Western blots of SDS-gel seperated nucleolar proteins stained by the same silver method used for AgNOR protein staining in cytohistological samples, the quantity of nucleolin and protein B23 has been evaluated in regenerating rat hepatocytes after partial hepatectomy and it was observed that their quantitative changes followed that of total AgNOR proteins.

These observations indicated that the quantitative differences of interphase AgNORs between neoplastic, hyperplastic and normal cells should be considered to be the consequence of a different number of proliferating cells present in the tissue. Support to this point of view was given by a series of results indicating a close relationship between interphase AgNOR amount and proliferative indices determined by different parameters of cell kinetics. Indeed, it was observed a positive relation between interphase AgNOR value and the percentage of S-phase cells determined either by means of DNA flow cytometry or incorporation of bromodeoxyuridine; interphase AgNOR quantity was also related to cell proliferation rate determined by Ki 67 immunostaining (Derenzini and Trerè, *Virchows Arch [B]* 61:1, 1991). However, if different amount of interphase AgNORs were merely due to a different number of proliferating cells, they should be very similar in all the cancers considered even if they had different mean interphase AgNOR values. This was absolutely not the case: among cancers of the same type (for example infiltrating ductal carcinoma of the breast) the greatest interphase AgNOR values were highly variable fom patient to patient, and related to the mean interphase AgNOR value. It appeared therefore that the quantity of interphase AgNORs (or AgNOR proteins) was not exclusively related to the number of proliferating cells, but other factors should influence it.

A possible explanation for the different distribution of interphase AgNORs in cancer lesions could be the different ploidy status of the cancer cells. Hyperdiploidy is the most frequent change in chromosome number in cancer cells. According to the evidence that the number of the total chromosomes is correlated to the number of acrocentric chromosomes (those, that is, carrying the NORs) the interphase AgNOR value could be also influenced. However, studies carried out on many types of human cancer cell lines have failed to demonstrate a significant correlation between interphase AgNOR value and DNA content (Derenzini et al. *Exp Cell Res* 211:202, 1994).

The explanation for this high variability of interphase AgNOR values in cancer lesions has been found considering the doubling time of cancer cells. Using human cancer cell lines characterized by different doubling times it has been clearly demonstrated that interphase AgNOR value and rapidity of cell proliferation were linearly related (Derenzini and Trerè, *Virchows Arch [B]* 61:1, 1991). A linear correlation was also found between the amount of nucleolin and protein B 23 and the cell doubling time. The different quantitative distribution of interphase AgNORs in rapidly and slowly proliferating cells can be explained considering the role of these structures and the AgNOR proteins in rRNA synthesis. A rapidly dividing cell must concentrate its ribosomal biogenesis in a shorter time than a slowly dividing cell. This can be achieved by activating a greater number of rDNA sequences for transcription. For this reason, a greater quantity of AgNOR proteins must be synthesized, which will give rise to a greater number of interphase AgNORs, the structural-functional units for rRNA synthesis.

The relationship between interphase AgNOR quantity and rapidity of cell proliferation represents the rational basis for the use of the AgNOR number in tumour pathology. The importance of interphase AgNOR quantification for diagnostic purposes appears to be reduced. Some neoplasms may be characterized by cell cycles with the same length as that of the corresponding hyperplastic lesions and therefore exhibiting similar interphase AgNOR values. Indeed, there were few types of cancers

in which there was no overlapping of interphase AgNOR values between malignant and benign lesions (Crocker, *Curr Top Pathol* 82:91, 1990), and the interphase AgNOR could then be recommended for diagnosis of malignancy. However, interphase AgNOR quantification, being the only method that permits information to be obtained on the rapidity of cell proliferation in samples processed for routine cyto-histopathology, can be used as a powerful variable in cell kinetics which is useful for diagnostic purposes.

Standardization of DNA quantitation by flow cytometry

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Owing to increasing applications of flow cytometric DNA measurements in oncology for the determination of ploidy and S phase fraction, standardization and quality control have become important. The data generated by flow cytometry must be precise and accurate to guarantee the reliability of the diagnostic and prognostic conclusions. They have to be reproducible to ensure the comparability of data yielded in different laboratories. This is especially necessary when rare tumors need to be analysed and data from various laboratories to be pooled for statistically significant evaluations.

During the last years three collaborative studies have been performed under the aegis of the Gesellschaft für Zytometrie, with the intention of obtaining an overview and appreciation of the preparation, staining and measuring methods used in our own and in neighbouring countries. As a result of this evaluation, it is intended to recommend the most accurate and reproducible methods for use and to establish standardization and quality control measures.

Samples of ethanol-fixed cells were distributed to the participating laboratories, and the participants were asked to stain and to measure the cells according to their routine procedure and to report the methodological details and results. The reports showed that two principal methods of staining and measuring predominate:

1. Staining with propidium iodide (mostly according to the Vindelov protocol or to modifications of this method) and measurement with an argon-laser-based flow cytometer,
2. Staining with DAPI (frequently according to the procedure published by Otto et al.) and measurement using instruments equipped with a mercury high pressure arc lamp (HBO).

Many laboratories did not follow the original protocols but developed their own modifications. To some extent this impaired the intention of the study, namely to evaluate comparable data from different laboratories. Other methods, e.g. staining with Hoechst or ethidium bromide+mithramycin, were used only occasionally. These can be omitted in future.

The comparison and evaluation of the data were performed with respect to the precision of measurement, the reliability of DNA index determination, and the reproducibility of S phase definition. The staining and measuring preciseness, as judged by the coefficient of variation (CV) values, proved to be extremely different among the laboratories. The intrainstitutional variability was smaller. Altogether, the combination DAPI staining and HBO lamp produced the most precise measurements, with CV values smaller than 1% in some samples and laboratories.

The DNA indices obtained from the histograms of aneuploid specimens also showed considerable variability. At least in part this can be attributed to insufficient measuring accuracy, lack of staining stoichiometry, and nonlinearity in data acquisition and processing. Similar results have been published by other groups (Wheeless et al., *Cytometry* 12:405, 1991; Danesi et al., *Cytometry* 14:576, 1993). Since the DNA indices are utilized for the characterization of aneuploid tumor cell clones, those variations are unacceptable and the roots have to be abolished.

The calculation of S phase fractions was considered to be prone to errors. Thus, the variability of the results reported by the various laboratories was not unexpected. It was conformable to the results of another inter-institutional study performed in Italy (Silvestrini et al., *Cytometry* 18:11, 1994).

A more detailed analysis of our results revealed consistent regularities. Frequently laboratories which calculated a low S phase fraction from one sample generated low S phase fractions also from the other samples, and, vice versa, laboratories which yielded a high S phase fraction from one sample brought out high S phase fractions also from the other samples. At first glance, this seemed to imply the existence of differences in the various computer programs used. But it turned out that this kind of regularity was more closely correlated with the laboratories than with the programs. This means that the operator who can influence the interactively working program mostly leads it unintentionally in the same direction thus producing biased data.

To answer the question as to whether the calculation of S phase fractions is influenced by the measuring accuracy, the deviations from the average S phase fraction were compared with the CVs. This comparison demonstrated that a correlation between the CVs and deviations from the average percentage of S phase cells exists. High CV values are prone to produce deviating S phase fractions.

The following conclusions can be drawn from the results of the first three collaborative studies:

At present, the intra- and particularly the inter-institutional variability of results obtained from flow-cytometric DNA measurements is high. The ability to compare and transfer data like DNA indices and S phase fractions between laboratories is rather limited. Further efforts of

standardization and quality assessment are still needed to establish flow cytometry as a useful and reliable tool in clinical oncology.

Image analysis versus flow cytometry

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Remarks on history and historical relation of the techniques

FCM dates back to 1934, where the initial effort was in the medical field, i.e. counting of blood cells (Moldavan, Science 80:188, 1934). From this time flow cytometry has developed to a very wide application in hematology as well as in other areas like biotechnology, microbiology, pharmacology, botanics, immunology and genetics. The technology obtained special support by the development of antibodies (Coons and Kaplan, J Exp Med 91:1, 1950) as well as the application of fluorescent dyes for the detection of DNA (Böhm and Sandritter, In: Evans (ed) *Cytology automation.* Livingstone, London, 1970), multiplying the options for analysis and enabling synchronous multiparameter analysis of different cell features. ICM has been invented in the 1960s, and the ideas were derived from needs in the aerospace and defence industries (for review: Sheldon, *Byte* 12:143, 1987) with applications in robotic vision and automated inspection. About 5 years later increasing interest in the medical field arose starting with questions on automated interpretation on cervical smears (for review of this time: Wied and Bahr (eds) *Automated cell identification and cell sorting.* Academic Press, New York, 1970), followed by the measurements of DNA by Böhm and Sandritter (1970).

We deal with two technically different methods that have different strength and weaknesses. ICM includes automated or semiautomated computer-based methods in which image information is digitized, stored, and subjected to quantitation of image features. A vast array of cellular measurements are possible, and many features like size or optical density are measured directly, whereas others as shape factors or ratios can be further extracted from adequate software provided with the instrument. The ICM pattern recognition uses feature extraction and optical density measurements as main tasks for subject characterization. Feature extraction procedures are peculiar to image analysis.

FCM, however, uses only crude information on cellular structure by measuring light scatter signals of cells passing a laser beam in a microscopic jet of water. The current flow cytometers are generally capable of measuring two light scatter and three fluorescent signals for a total of five features per cell. Each cell produces a short flash of light, the intensity of which is proportional to the cellular content of the fluorescently labeled constituent. After adequate optical filtering these flashes are transformed into electrical pulses and amplified and stored and further analysed with computers.

Nevertheless, both methods find their domains in clinical applications because of their technical advantages (see below). Where both methods seem equally valid as a diagnostic complement, we suggest both methods to be used in parallel to determine their validity, in prospective studies.

Current status of clinical application

Weinberg has recently summarized the current applications for clinical routine (Weinberg, in: Bauer et al. (eds) *Clinical flow cytometry,* Williams & Wilkins, Baltimore, 1993).

Cell surface marker analysis is certainly the most frequently performed test in most clinical FCM laboratories, and is best performed using this technology. As one example the immune status (especially the CD4/CD8 ratio) of blood or bronchiolar lavage can be named. DNA content and cell proliferation assays can be and have been performed with both techniques for about 25 years. However, looking at it critically, we find a strong discrepancy between its very sparse clinical application and numerous studies showing promising results with ploidy or S-phase fraction (SPF) determination as prognostic factors (Shankey et al., *Cytometry* 14:472, 1993). Resulting from the awareness of this discrepancy, increasing efforts are being made to establish standardization protocols, in order to validate the methods in prospective studies and to determine inter- and intralaboratory variability. In ICM task force groups on standardization of DNA (Böcking et al., 3rd Conf of ESACP, Grenoble, 17 May 1994) and AgNOR measurements (Aubele et al., *Zentralbl Pathol* 140:107, 1994) have to be mentioned, whereas similar attempts in FCM include the efforts of the New York Bladder Cancer Flow Cytometry Network Study (Wheeless et al., *Cytometry* 12:405, 1991) and the DNA consensus meeting held in New York in October 1992 (Hedley et al., *Cytometry* 14:471, 1993).

Perspectives of both methods for clinical routine

While the efforts for standardization are a mandatory part for the application in the clinical situation, another interesting approach to improving the clinical statement is the direct comparison of the two methods. Looking at studies comparing FCM and ICM directly, DNA content measurements are nearly exclusively used for comparison (Falkmer et al., *Anal Cell Pathol* 2:297, 1990; Kaern et al., *Cytometry* 13:314, 1992). So far most of the studies use fairly low sample numbers (<100), and data are derived from unimodal DNA assessment (Leung et al., *Mod Pathol* 7:195, 1994, Berner et al., *Anal Cell Pathol* 5:339, 1993). Thus the main conclusion drawn in a num-

ber of studies, ICM-DNA being more sensitive in detecting aneuploidy in the near diploid region, might not be valid if the advantage of marker selection for tumor DNA is applied in FCM. However, no studies can be cited on this topic yet.

Further, we doubt the reliability of SPF determination by ICM, knowing the necessity for high cell numbers for reliable cell cycle algorithms. However, another area of proliferation assessment is very promising for ICM, i.e. the detection of proliferation associated antigen that can be quantified under consideration of topography (Schwartz et al., *Am J Pathol* 134:327, 1989; Knüchel et al., *Virchows Arch [B]* 64:137, 1993) and should be used and compared with FCM to determine the value of this methodology.

Within the context of optimizing the methods for clinical use, efforts at further instrumentation of specific technology also have to be mentioned. ICM will support and is partly supported by neural networks, representing a non-algorithmic method of generating computer learning. Such systems are especially adept at tasks involving image recognition and classification (Roberts, *Science* 243:481, 1989), and will result in high-speed recognition. Attempts to get over the hurdle of artefact discrimination consist in advances in specimen preparation, scene segmentation and artificial intelligence systems.

In FCM algorithms for debris and doublet discriminations are becoming increasingly sophisticated. Also, cell cycle analysis is improved in order to overcome the interactive step of defining G0/G1 and G2/M channels. As an important practical effort the application and standardization of dual parameter analysis results in more precise (tumour-selective) assessment of the SPF (Visscher et al., *Lab Invest* 62:370, 1990; Knüchel et al., *Recommendations on a consensus protocol for dual parameter flow cytometry,* in preparation).

To round off the spectrum of promising and partly synergistic application of ICM and FCM in the medical field a direct combination of the two methods is recomended. Clinical application is already practiced by applying multiparameter phenotyping of nucleated erythrocytes which are subsequently isolated by a cell sorter and checked for trisomies by applying fluorescence in situ hybridization (FISH) on the sorted cells (Simpson and Elias, *JAMA* 270:2357, 1993).

Evaluation of new prognostic factors in oncology

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Definition of prognostic factors in oncology

The outcome of patients with malignant tumours is influenced by a variety of factors. Conventionally, each individual putative factor is primarily isolated and examined in relation to outcome. Because there is a multitude of interactions of different strengths among the different factors, univariate analyses always have to be followed by multivariate biometric analyses. These techniques allow the identification of factors with independent influence on outcome and the quantitative assessment of the influence by estimating the relative risks associated with each covariate. Thus, in oncology "prognostic factors" should be defined as variables or covariates with independent influence on outcome.

Prognostic factors and outcome definitions

Prognostic factors may be different according to the various ways of measuring outcome, e.g. overall survival, adjusted (cancer-related) survival, disease-free survival, relapse rate (locoregional and/or distant recurrence), response to treatment.

Prognostic factors for different patient subgroups

Prognostic factors may be effective in all patients or only in some subgroups, e.g. specific TNM or pTNM categories or stages or patients with specific treatment modalities. Generally, prognostic factors are different also for patients with no residual tumor after initial treatment (R0) and those with remaining residual tumour (R1, 2).

For the individual tumour sites and entities, there are proven prognostic factors. These were identified by appropriate multivariate analyses of large data sets from at least two separate studies and generally accepted in clinical practice. For most solid tumours, anatomical extent of disease, as assessed before treatment by the TNM and pTNM classification and after treatment by the residual tumour (R) classification, is the most important proven prognostic factor. However, other tumour-related, patient-related and treatment-related factors are important for prognosis. As treatment improves, the importance of a given prognostic factor may change, and new analysis of the prognostic factors may be necessary.

Acceptance of new prognostic factors

New technologies in oncological diagnosis and the respective findings necessitate the analysis of putative new prognostic factors. This has to be performed against the established knowledge on prognostic factors. Before new prognostic factors can be accepted and widely used in clinical research and patient management, five requirements have to be met: (1) The method of determine the factor must give good reproducibility, i.e. the methods have to be standardized and inter- and intra-observer variability has to be low. (2) The significance and independence of the putative factor must be demonstrated by multivariate analyses of data from at least two institu-

tions. It should be tested on another independent data set. (3) The new factor must provide additional prognostic information beyond that provided by such conventional factors as anatomical extent, grade or gender. (4) The effect of the new prognostic factor has to be specified in regard of the end point; it also has to be clarified whether the factor is effective for all patients or only for some subgroups, e.g. TNM/pTNM categories, specific treatment, etc. (5) The assessment of the new factor must be possible in the majority of institutions treating the specific tumour entity and the costs have to be proportionate to the benefits.

Problems in prognostic factor analysis

First of all, it has to be emphasized that at present there is no universally accepted biometric method applicable to all circumstances. Furthermore, results of multivariate analyses are influenced by: the selection of analysed data; the proportion of missing data; the chosen end-points; the number of patients and their selection; differences in data collection.

Thus, results of prognostic factor analyses are valid only for data sets with the same patient structure and the same data collection method. Therefore, assessment of a given prognostic factor by data splitting or cross-validation and testing on new independent data are essential.

Importance of prognostic factor knowledge

In clinical practice, the knowledge of prognostic factors is important to understand the course of disease; to predict the outcome for the individual patient; to alleviate patient anxiety; to select appropriate treatment modality; to explain variation in treatment outcome; to plan specific therapeutic intervention. The objectives of prognostic factors in clinical trials are to characterize and predict patient outcome; to examine interrelationships and degree of dependence among important factors; to ensure comparability of patient groups in randomized trials; to allow more precise analysis of the differences in outcome; to identify subgroups for novel treatment approaches; to serve as an intermediate endpoint in early detection.

Further development

Prognostic index and prognostic systems. Studies of prognostic factors offer the possibility of creating mathematical models, including prognostic indices that predict the individual overall, adjusted and/or disease-free survival and/or the individual risk of locoregional and distant recurrence for each patient on the basis of the clinical and pathological presenting condition and the form of therapy used. Then ultimate goal is to provide a so-called prognostic system, i.e. an accurate system of predicting outcome for individual patients so that physicians can plan therapeutic interventions at any time during the course of disease.

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Proliferative measurements in breast cancer

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Aims

Our studies of proliferation-related markers in clinical breast cancer have been largely restricted to measurement of S phase fraction (SPF) from flow cytometric DNA histograms. Most of this work was made using archival, paraffin-embedded tissue (Camplejohn RS, Ash C, Gillett CE et al., *Br J Cancer,* in press) but we have also done some measurements on unfixed cells obtained by fine needle aspiration (FNA). Both these aspects of DNA flow cytometry will be discussed as well as the possibility of performing dual fluorescence measurements of DNA content plus bromodeoxyuridine incorporation. Finally, a brief discussion of the measurement of DNA content in combination with proliferation-related proteins, such as Ki-S1 antigen, will be made.

Methods

We have performed a number of DNA flow cytometric studies of breast cancer, in which the prognostic value of S phase fraction (SPF) and DNA ploidy was investigated. The largest of these investigations, performed on archival, paraffin-embedded tissue, was a single-centre study of 881 patients, in which SPF was shown to be a significant prognostic marker in terms of overall survival (OS), relapse-free survival (RFS) and survival after relapse (SAR). Further, the prognostic power of SPF was independent of a range of other clinicopathological variables, namely, tumour grade and stage, nodal status, patient age, tumour size, menstrual status and treatment details. For OS and RFS, SPF was the second strongest predictor of the clinical course of the disease after nodal status, and for SAR it was the strongest prognostic marker. SPF correlated positively with histological grade but was the stronger predictor of survival. The distribution of SPF values was markedly different for the two ploidy classes of tumour, with DNA aneuploid tumours having a significantly higher average SPF. However, SPF re-

tained its independent prognostic ability when DNA diploid and aneuploid tumours were analysed separately. DNA ploidy itself also proved to be an independent prognostic marker but the survival difference between the two ploidy classes was much less than that seen for different levels of SPF. Tumours with several DNA aneuploid populations (multiploid tumours) tended to have a worse prognosis than other aneuploid tumours but this trend did not reach statistical significance. In this and other studies from this centre, SPF has proved to be a robust predictor of clinical outcome in carcinoma of the breast. Perhaps the most interesting finding regarding SPF was that it appears to have the potential for selecting patients for adjuvant chemotherapy. We are currently investigating this finding further.

Fine needle aspiration (FNA) has frequently been used for the diagnosis of breast malignancy. It can be a highly accurate procedure in the hands of a skilled cytopathologist. We have compared DNA flow cytometry carried out on FNAs with that performed on archival, paraffin-embedded tissue. The quality of the DNA histograms from aspirates was very good, with an average CV of 3.7. Comparison of the breast aspirates with paraffin-embedded sections from the same tumours showed good agreement in terms of ploidy and S phase fraction. There are many advantages to fine needle aspiration of breast tumours, as it is a simple, rapid technique which offers the possibility of measuring other parameters combined with DNA content.

A disadvantage of most proliferation-related measurements that are clinically feasible is that they only give a static picture (Camplejohn, *Ann Oncol* 4:184, 1993); they do not give truly kinetic information. One method that does yield real cell kinetic data, is the DNA/bromodeoxyuridine (BrdUrd) technique. In this method, the progress of BrdUrd-labelled cells around the cell cycle is measured flow cytometrically, yielding an estimate of potential doubling time (Tpot) and S-phase duration (Ts). We have not been able to apply this method to breast cancer but others have done so (Rew et al., *Br J Surg* 79:335, 1992).

Much of our work in recent years has been on methods of measuring proliferation related proteins combined with DNA content. Most of these studies have been aimed at basic biological or technical questions (Wilson et al., *Eur J Cancer* 28A:2010, 1992) rather than clinical ones. However, a collaboration with Dr. Kreipe on Ki-S1 did include a small amount of work on clinical breast cancer material (Camplejohn et al., *Br J Cancer* 1993). In this study we: (1) defined the pattern of Ki-S1 labelling relative to the cell cycle phase; (2) investigated the labelling pattern with Ki-S1 on a human breast cell line (ZR75) under varying proliferative conditions induced by serum deprivation and refeeding; (3) examined in a flow cytometric study Ki-S1 staining in archival, clinical breast carcinoma samples. In exponentially growing cells Ki-S1 showed a marked cell cycle phase-specific variation in staining intensity which increased linearly through the S phase, was high in G2 and reached its peak in mitosis. Ki-S1 staining intensity mirrored the changes in proliferation activity of ZR75 cells during serum deprivation and refeeding. In a small series of human breast carcinomas, Ki-S1 staining intensity correlated with S-phase fraction (SPF) derived from DNA profiles. The antigen labelled by Ki-S1 is extremely robust, resisting degradation by fixation and by an aggressive enzymic tissue disaggregation method. It was felt that Ki-S1 warrants further investigation as a proliferation-related marker, particularly for routine clinical application.

Interpretation of proliferation markers

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Introduction

By the end of the 1960s, the extensive use of tritiated thymidine resulted in a mathematical description of cell population kinetics (Cleaver, *Thymidine metabolism and cell kinetics,* in: Neuburge and Tatum (eds) *Frontiers of biology* 6, North Holland, Amsterdam 1967). Although these equations of cell proliferation kinetics offered a well-established and non-controversial framework in which to interpret proliferation markers, they have been progressively neglected with the overwhelming panel of tumour markers becoming widely available in biological and clinical research. This paper intends to re-introduce 'common sense' in the interpretation of proliferation markers in order to more closely define what proliferation markers tell us about proliferative activity.

Proliferation activity

Proliferative activity can be defined according to its mechanisms or its result. The mechanisms responsible for proliferative activity are the speed of the cycle which is inversely proportional to the *generation time (T)* on the one hand, and the proportion of cells committed to cycle, or *growth fraction (G),* on the other. Thus, the *proliferative activity (P)* is defined as $P=G*1/T$. It is clear from this equation that neither the growth fraction nor the generation time can alone define the actual proliferative activity of a tumour. Although the cell cycle duration can readily be assessed in vitro, in clinical practice the cell cycle time is not accessible and thus the proliferative activity cannot be measured.

Proliferation markers

Growth fraction (Ki67)

The Ki67 monoclonal antibody introduced by Gerdes et al. (*Int J Cancer* 31:13, 1983) demonstrates cells at any

step of the cycle and has provided, for the first time, a direct means of evaluating the growth fraction of tumours in histopathology and cytopathology. The growth fraction, assessed by Ki67 labeling index, is the occurrence rate of the cycling cells, i.e. number of Ki67 positive cells/number of Ki67 positive plus the number of Ki67 negative cells. In practice, some underestimation of the growth fraction may occur due to the rapid disappearance of the antigen in post-mitotic cells.

Cell cycle phase recognition (BrdU, PCNA)

Apart from the M phase, which can be recognized in conventional preparations, the only cell cycle phase that can be specifically identified is the S phase, owing to incorporation of either isotopic DNA precursor (tritiated thymidine), or thymidine analogue, such as bromodeoxyuridine (BrdU). Another way to detect specifically S phase cells is to label the DNA polymerase bound to DNA. The proliferation-associated nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase (Bravo et al., *Nature* 326:515, 1987). This protein is synthesized during G1 phase; its amount is maximum during S phase but becomes very low at mitosis and in non proliferating cells as well. The interpretation of the amount of PCNA to decide whether a cell is in cycle or not is difficult. Moreover, the labelling can be impaired by the preparation techniques. PCNA is thus not a suitable alternative to Ki67. Nevertheless, there is a fraction of PCNA strongly bound to the DNA replication sites, which is not sensitive to the fixation procedure and the antibody used. This stable labelling can be specifically recognized by a punctuated nuclear pattern, in contrast to the PCNA weak fraction, which appears as a diffuse labelling over the nucleus and the cytoplasm. The very reliable punctuated labelling of PCNA has proved to be identical to the labelling pattern obtained with BrdU (Humbert et al., *J Cell Sci* 103:97, 1992). Therefore, PCNA is a reliable S phase marker which appears as the method of choice to evaluate the S phase index in histopathology (Galand and Degraef C, *Cell Tissue Kinet* 22:383, 1989).

Phase index (SPF, MI)

Phase index is the frequency of cells in a phase of the cell cycle that can be specifically recognized (mitosis) or labelled (S phase). The proportion of cells in a cell cycle phase results only from the relative duration of that phase in the cycle (phase duration/cycle time) and the growth fraction. Since $SPF=G*S/T$ and $P=G*I/T$, then $SPF=P*S$ and similarly $MI=P*M$. From this formulation, several statements can be inferred:

1. If two tumours have a different phase index (MI or SPF), this may result from a difference in: growth fraction while their respective phase duration to cycle time ratio is similar, or in the phase duration to cycle time ratio while their respective growth fraction is similar, or in the absolute phase duration while their respective proliferation activity is similar, or in proliferative activity while their phase duration is similar. The analysis of the phase index does not make it possible to decide which of these possible causes applies. Between two tumours having different phase index (say, SPF), the one with the higher value is not necessarily the one that has the most active proliferation.
2. There is no biological rationale that allows us to expect any systematic correlation between the growth fraction and any phase index.

Unfortunately, the conclusions made in about a quarter of the clinical studies involving proliferation markers (MI, PCNA, BrdU, Ki67...) published in the literature are in flagrant contradistinction with at least one of these unescapable statements and have thus to be disregarded.

Cell cycle time (AgNOR)

Cell cycle time is definitely the variable that is missing from the attempt to interpret differences and similarities between tumours in terms of cell proliferation kinetics properly. The cell demand for RNA during the cell cycle is particularly high, to ensure both cytoplasmic growth and the structural and functional proteins of DNA replication. This results in amplification and over-activity of the ribosomal genes that can be revealed by associated argyrophilic proteins known as AgNORs (Derenzini et al., *J Histochem Cytochem* 38:1237, 1990; Ploton et al., *Histochem J* 18:5, 1986). In malignant cells, the nucleolar organizer regions are increased in number and clustered when compared to benign cells (Crocker et al., *J Pathol (Lond)* 158:185, 1989). The relationships between cell cycle and AgNOR were reported through a series of studies demonstrating that the AgNOR quantity increases during G1 to reach a maximum during S, this quantity being inversely proportional to the cell cycle duration: the faster the cycle, the higher the amount of AgNOR (Derenzini and Pession, *Am J Pathol* 1134:925, 1991; Derenzini et al., *Lab Invest* 63:137, 1990).

Evaluation of tumour kinetics

Since the amount of AgNORs increases with the cell cycle time, it is a direct measurement of $1/T$ in arbitrary units, whereas Ki67 labelling index is a measurement of G. The proliferative activity can thus be simply expressed as P=Ki67*AgNORs. Owing to this equation, tumours can be compared within a conventional 2D graph, where the populations having the same proliferative activity are distributed along an hyperbola as illustrated in Fig. 2.

Averaging AgNORs values (cycle speed) as well as Ki67 labeling index (growth fraction) and PCNA or

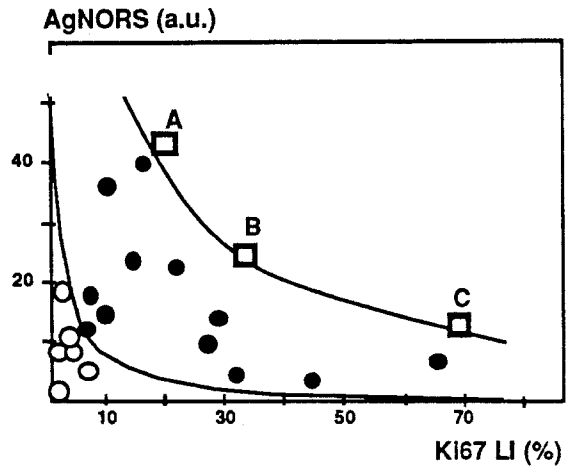

Fig. 2 Scattergram of 6 normal epithelia (white rounds), 12 non-invasive (black rounds) and 3 invasive (white squares) transitional carcinomas of the bladder. The tumours having the same proliferative activity (P=Ki67*AgNORs) are distributed along the same hyperbola as observed for invasive tumours A, B and C. Interestingly, no Ki67 or AgNORs threshold alone can discriminate between normal, non-invasive and invasive cases but the combination actually can.

BrdU labeling index (S phase index) over a large area of the tumour to attain statistical significance may result in minimizing the high values observed in local areas where both the growth fraction and the cell cycle speed are high and which may have a real prognostic significance. Methods must now be developed to take account of the distribution of proliferating cells and represent tumour heterogeneity by quantitative and reproducible features.

Single-cell-based quantification of the proliferation fraction by image analysis

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The most common antibodies in use are Ki-67 (MIB-1) and PCNA, both binding to nuclear structures of proliferating cells (Bravo et al., *Nature* 326:515, 1987; Gerdes et al., *J Immunol* 133:1710, 1984). The unrecognized Ki-67 antigen, most probably an element of the nuclear matrix, is present between the early G1 phase and mitosis; this means that the Ki-67-antibody, which can be applied only on frozen sections, and its equivalent MIB-1, that can be used also on paraffin sections, are markers for all cells in the cycling pool (for details see: Kreipe et al. in this report).

The evaluation of immunostaining results has up to now most often been performed by subjective semiquantitative estimation of the rate and the staining intensity of the immunolabelled nuclei. This method, however, is somehow rough and of limited interobserver reproducibility (Biesterfeld et al., *Cancer Res* 1994). As an alternative, image analytical techniques can be applied; methodologically, a conventional indirect peroxidase technique is used with DAB serving as chromogen and methyl green as counterstaining.

For image analysis, a TV cytometer CM-2 (Hund, Wetzlar, Germany) is used (Möller HW et al. *Compendium of the computerized cytology and histological laboratory,* University of Chicago Press, Chicago, pp 376–388, 1994).

The hardware is composed of a Hund microscope H 500 LL with a filter wheel between light source and microscope, armed with a red interference filter (wavelength: 623±5 nm) and a green interference filter (wavelength: 550±10 nm). Further hardware components are a CCD camera, a high-resolution monitor and a modular TV image analysis system based on mathematical morphology.

Image analysis is performed using a new software package that has been developed in close cooperation with our institute. With this, for the first time results of immunostaining can be calculated as a function of the number of nuclei and not as a function of the nuclear sum area.

The rate of immunolabelled nuclei is calculated in three steps. At first, the microscopical image is digitized at the two different wavelengths, the red filter representing an optimal contrast for the methyl green-stained nuclei and the green filter representing an optimal contrast for the DAB-positive nuclei. Then, the red image is segmented by a watershed algorithm in order to find the individual masks of all tumour nuclei; if necessary, corrections can be performed interactively using special software applications. Latest, the segmentation is transferred to the green image, and the rate of immunostained nuclei is calculated as the quotient of counted nuclei in the green image compared with the red image. After measurement of at least 40 viewing fields with 20× objective magnification, several statistical parameters are calculated, including a staining intensity histogram.

Preliminary results from methodological studies on astrocytomas, meningiomas and carcinomas of the prostate indicate a satisfactory correlation between image analytical proliferation assessment and semiquantitative estimation. The distribution of immunostained nuclei within the viewing fields appeared to be quite heterogeneous, according to increased 95% confidence limits. The rate of immunostained nuclei was not correlated to DNA cytometric parameters.

Finally, it should be pointed out that the same image analytical technique can also be applied to other nuclear binding immunoreactions, e.g., for oestrogen and progesterone receptor analysis.

Clinical significance of nucleolar organizer region – assiociated proteins in tumour pathology

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Technical considerations concerning AgNOR staining and quantitation

Since the colloid silver-staining technique for visualisation of AgNORs has been widely applied in histopathology, several groups have indicated that staining results are substantially influenced by the fixatives used (Smith et al., *J Pathol (Lond)* 155:109, 1988; Griffiths et al., *J Pathol (Lond)* 159:121, 1989, Öfner et al., *Pathologe* 15:226, 1994). In contrast to tissues processed with alcohol-based fixatives, formalin-fixed and paraffin-embedded specimens were shown to produce poor staining quality, with AgNORs not being distinguishable as substructures of the nucleolus. Astonishingly, in the plethora of studies performed in recent years, few have taken these limitations into account (Öfner et al., *J Pathol (Lond)* 162:43, 1990; Mourad et al., *Hum Pathol* 24:206, 1993). Morphometric analysis was proposed to overcome these shortcomings (Rüschoff et al., *J Pathol (Lond)* 161:113, 1990; Derenzini and Trerè, *J Pathol (Lond)* 165:337, 1991). Recent work from our group has shown that quantitation of AgNORs either by eye or by morphometric analysis is considerable influenced when evaluation is performed on grounds of an improved silver staining quality obtained after wet autoclave pretreatment of sections (Öfner et al., *Mod Pathol* 7:946, 1994). These inconsistencies regarding staining quality and quantification methods applied have led to widely divergent AgNOR quantities within a given tissue (Trerè, *Zentralbl Pathol* 140:11, 1994). Therefore it appears that results obtained in studies performed on formalin-fixed and paraffin-embedded tissues are not comparable. Nevertheless, particularly in the light of our improved staining regimen, AgNOR analysis and its clinical implications will probably be an important tool in diagnostic histopathology and clinical management of appropriate patients.

Diagnostic value of AgNOR analysis

After 8 years of systematic evaluation it is acknowledged that the quantity of interphase AgNORs in cancer cells exceeds those in their benign counterparts. Nevertheless, the AgNOR content has been reported to show considerable overlap between malignant and benign cells, which makes this variable rather useless as a criterion of malignancy. Only in pleural and peritoneal effusions was a well-defined cut-off in the AgNOR values reported between reactive and malignant mesothelial cells (Ayres et al., *Thorax* 43:366, 1988; Derenzini et al., *Acta Cytol* 33:491, 1989; Barsotti et al., *Diagn Cytopathol* 6:289, 1990; Lim et al., *J Pathol (Lond)* 166:53, 1992).

Prognostic value of AgNOR analysis

Since evidence is increasing that the AgNOR content of tumour cells is strictly related to both the percentage of cycling cells and the speed at which cells proliferate (Derenzini et al., *Histochem J* 24:951, 1992; Öfner et al., *Pathol Res Pract* 188:742, 1992) retrospective studies have been carried out to evaluate the predictive value of AgNOR in tumour pathology. From all these studies it can be concluded that a majority of investigations have proven the prognostic value of interphase AgNORs. In particular non-Hodgkin lymphoma, breast carcinoma, and colorectal cancer had been intensively investigated by several groups. Despite the lack of standardised staining and quantification procedures, almost all studies revealed a positive correlation between the AgNOR content, various proliferation markers (Ki-67, PCNA, SPF, etc.) and patient survival. Moreover, AgNOR variables had been proven to predict survival as independent variables in these tumours (Rüschoff et al., *Pathol Res Pract* 186:85, 1990; Jakic-Razumovic et al., *J Clin Pathol* 46:943, 1992; Jakic-Razumovic et al., *Leuk Lymphoma* 7:165, 1992; Joyce et al., *Ann R Coll Surg Engl* 74:172, 1992; Lesty et al., *Anal Quant Cytol Histol* 14:175, 1992; Aubele et al., *Path Res Pract* 190:129, 1994). A similar independent predictive value has been found in renal cell carcinoma (Delahunt et al., *J Pathol (Lond)* 170:471, 1993), multiple myeloma (Pich et al., *Virchows Arch [A]* 421:143, 1992), soft tissue sarcoma (Tomita et al., *Int J Cancer* 54:194, 1993), carcinoma of the prostate (Contractor et al., *Urol Int* 46:9, 1991), urinary bladder carcinoma (Lipponen et al., *Br J Cancer* 64:1139, 1991), adenocarcinoma of the lung (Abe et al., *Jippon Kyobu Shikkan* 29:1282, 1991), squamous cell carcinoma of the head and neck (Pich et al., *Br J Cancer* 64:327, 1991), oesophagus (Morita et al., *Cancer Res* 51:5339, 1991), and carcinoma of the cervix uteri (Tosi et al., *Pathol Res Pract* 188:866, 1992).

Although these findings are encouraging it has again to be stressed that further studies based on standardised staining protocols and quantification methods are necessary to reliable confirm the clinical value of AgNOR analysis.

Guidelines of AgNOR quantification – first update

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On the occasion of the second international workshop on AgNOR quantification in Regensburg experiences with staining and evaluation of AgNORs have been discussed extensively. Since our first meeting (Berlin, 1–3 October 1993) two contributions of major importance for standardization have been achieved. First, Öfner et al. (*Mod Pathol* 7:946, 1994; *Pathologe* 15:226, 1994) have introduced the wet autoclave procedure to the silver staining protocol of routinely formalin fixed specimens. This modification improves the staining of AgNORs dramatically. Second, in order to determine those AgNOR variables which can be evaluated most reproducibly a multicentre study was performed. AgNOR-stained slides of 60 breast cancer specimens have been measured by four different laboratories (Trerè et al. *J Pathol (Lond)* 1995, submitted).

Based on these two studies and on the personal experiences of the Committee members using these techniques staining and evaluation of AgNORs in histological samples fixed in buffered formalin and routinely paraffin-embedded should be performed as follows.

Staining protocol

a. Mount the 2–5 µm sections on silane-coated glass slides
b. Dewax and rehydrate in graded ethanols in xylene
c. Immerse sections in sodium citrate buffer (0.01 M sodium-citrate monohydrate, pH 6.0)
d. Boil the sections in plastic Coplin jars at 120° C for 20 min (in an autoclave)
e. Cool down to room temperature and wash with distilled water
f. Dissolve one part of volume of 2% gelatine in 1% formic acid in two parts of 25% aqueous silver nitrate solution and stain in the dark at room temperature
g. Thorough washing with A.dest., dehydrate and mount as routine

Methods of evaluation

As pointed out in the first guidelines (Aubele et al., *Zentrbl Pathol* 140:107, 1994) image cytometry is the method of choice for evaluation. Thereby, total area of AgNORs per cell is the basic indicator of AgNOR quantity. According to the recent multicentre study, the coefficient of variation relative to the AgNOR protein area (CV=standard deviation of area per cell/mean area value) has been shown to be the most reproducible parameter of AgNOR protein quantity. This value should be determined in 100 tumour cells where microscope magnification is set at 40× (objective lens). All tumour cells of a given image are then measured in one focal plane.

Counting of AgNORs by eye is the method of second choice and should be restricted to those slides/or cells exhibiting distinct silver stained dots as definite substructures of the nucleoli.

Conclusions

Proliferation is one important feature which characterizes the malignant phenotype of neoplasia. During the last few years new mechanisms of cell cycle control and several markers of so-called proliferation associated factors have been identified. Concomitantly numerous papers have been published demonstrating the expression of such factors in human neoplasia.

It was the aim of the Regensburger Workshop to give an overview of the techniques used in the assessment of proliferation and to discuss their practical value in clinical oncology. The opinion of all experts was obtained by means of a questionnaire. According to the answers given, there was general agreement that proliferation assessment (1) is an important or at least complementary tool in clinical oncology, and (2) should be performed by the surgical pathologist. In contrast, it was estimated that in less than 5% of all cancer patients and in less than 10% of institutions equipped with the technical facilities was the proliferative activity of tumours assessed routinely. The reasons for such discrepancies between theory and practice were presumed to lie mainly in the field of standardization and data interpretation. The latter aspect was particularly emphasized by G. Brugal, who clearly showed that proliferation activity cannot be determined reliably by means of only one proliferation marker. With regard to the specific tumour groups the proliferation assessment is of proven prognostic significance in lymphoma, breast, prostate and urinary bladder carcinoma. The importance for therapeutic decision making is thought to be high for breast carcinoma, and less so for urinary bladder and prostate carcinoma. Finally, it has been pointed out by P. Hermanek that for the acceptance of proliferation assessment techniques as a possible source of clinically relevant prognostic factors specific rules have to considered. Apart from standardization of methodology, these include univariate and multivariate analyses, exact definition of the respective subgroups of patients, and definition of the different end-points in question, as well as the logistic and economic conditions.