Dysplasia in view of the cell cycle

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Dysplasia is linked to altered tissue architecture. The lesion belongs into the diagnostic field of human pathology and is highly relevant for the clinical physician, because it breaks the criteria of hyperplasia and regeneration. Dysplasia is a precancerous disorder leading in all probability to malignant transformation if not treated. However, different descriptions do apply for dysplasia in different human tissues, and conventional pathology cannot arrive at unequivocal stringency. In contrast to the previous situation, now, dysplasia is defined by a unifying concept, which works upon cell cycle criteria. The decisive element for the proposed definition is unbalanced segregation of chromosomes and persistent genomic asymmetry through telophase, leading to aneuploid interphase nuclei. Progress of dysplasia can be estimated from the frequency of pathologic mitoses that directly measure cellular proliferation. In routine work, progress of dysplasia shall be quantified by frequency increase of aneuploidy in the increasing fraction of proliferating interphase nuclei. Thus, dysplasia is defined not only by aberrations from healthy histological architecture and normal cytological differentiation, but also by violations of the DNA standard from mitotic nuclei. The proposed classification of dysplasia measures the frequency of pathologic mitoses and the degree of genomic alterations in interphase nuclei. Both these criteria discriminate between low-grade and high-grade dysplasia and ascertain the malignant potential of a dysplastic lesion.

Key words: aneuploidy, cell cycle, clonal selection, dysplasia, genomic instability, pathologic mitosis

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Epistemic Roots
Clinical experience competed with anatomical discriminations for higher reliability 115 years ago. Light microscopy enabled the histological pathology created by Rudolf Virchow (1885). His insight that cancers were built up by cells, paved the way for the microscopic description and classification of malignant tumours. They were understood to develop in healthy tissues.

Proliferation means that every cell originates from another cell (Virchow 1858). *Omnis nucleus e nucleo* (Hertwig 1876) represented a further rule for normal development as well as tumorigenesis. *Karyokinesis* subsumed the dynamic steps that divide the chromatic substance in the cell's nucleus. The process of nuclear division was opposed to the "resting" nucleus, which makes the chromosomes actually active (Boveri 1887). This period is now termed "interphase". We owe the concept of the cell cycle to Valentin Haecker (1892). Walther Flemming (1891) understood that every chromosome detaches from another one that had reduplicated. He explained that "initial splitting" as being different from the longitudinal separation during anaphase. David Hansemann, assistant to Virchow, reported on asymmetric divisions in epithelial cancers and their biological significance. He postulated that unbalanced anaphases lead to unequal chromatin distribution resulting in cancer. Hansemann (1890, 1891, 1897) confirmed his view, but did not exclude that asymmetric chromosome segregation might occur not only in cancers. Just at the beginning of the 20th century, the concept of tumorigenesis respected cellular and nuclear pathology.

Careful clinical observations and follow-ups referred to "precancerous conditions" that may precede the development of cancer. John T. Bowen (1912) published two cases of precancerous dermatoses due to chronic, atypical epithelial proliferation. The excellent micrographs showed abnormal transformation, cornification of rete cells and...
karyokineses. The author did not mention that the nuclear divisions showed any abnormality, but he expected that such lesions might readily assume a malignant type of cell growth. Furthermore, Bowen gave credit to Dubreuilh (1896) who had discussed several affections under the heading “precancerous keratoses”.

Four case descriptions upon atypical squamous epithelium of the uterine cervix considered in detail (1) the cellular morphology, (2) the pattern of epithelium and (3) its relation to glands and connective tissue. Schauenstein (1908) discussed precancerous lesions as incidental findings that could be important in pathological and in clinical respect. He mentioned also frequent asymmetric mitoses.

The central period of nuclear interphase received the persistent definition “S phase” (Howard and Pelc 1953) by the year revealing the structure of DNA. A mitotic S phase reduplicates the maternal and paternal complement so that the 7 pg DNA (6.8 Mbp) from a human G1 interphase nucleus increase to 14 pg. The latter amount persists during G2 up to the segregation in anaphase. The quantitative aspect may be short cut by saying that nuclear DNA synthesis reduplicates two complements into four: 2 x 2 c = 4 c. Hewson Swift (1950) coined the “C-value” that represents the (haploid) genome equivalent. The Stockholm school pioneered quantitative studies of nuclear DNA. One of these microphotometric investigations recorded aberrant DNA contents in interphase nuclei from cancers of the uterine cervix; but most interesting was the finding of similar aneuploidy in precancerous lesions (Caspersson 1964). The source of aneuploidy in interphase was detected by direct measurements of unbalanced telophases from a variety of precancerous lesions (Steinbeck 1997a).

The present essay provides a solid definition of premalignant tumours, generally named “dysplasias” in human pathology. Dysplasia is understood as an intermediate stage between normal epithelium and cancer, which is morphologically defined by a loss of normal cell orientation. Dysplastic cells are frequently deprived from normal control over proliferation. The amount of nuclear DNA represents the most important parameter to decide on a regular or disturbed performance of the cell cycle. Representative examples of quantitative DNA records of interphase nuclei as well as unbalanced divisions are shown from affected tissues. Such records from individual somatic nuclei allow judgments and prognoses about endangered patients. Critical examples of dysplasia were reported especially from mucous membranes of mouth, stomach and vulva. Such cases were chosen, since they are at risk of under-diagnosis or over-diagnosis. After routine examination by light microscopy, twin sections (10 µm for interphase nuclei and 15 µm for divisions) were Feulgen stained. Amounts of nuclear DNA in lesions were calibrated with endogenous lymphocyte nuclei indicating the 2 c level using an image processing microphotometer (Steinbeck et al 1999).

Old concept of dysplasia

Knowledge about dysplasia in the uterine cervix promoted the idea that an early detection and immediate treatment could prevent cancer. The close correlation between histological and cytological records paced the classification of premalignant lesions (Reagan and Hamonic 1956, Burghardt and Holzer 1970, Rlotton et al 1973). Since the basis of dysplasia remained unknown, competing classifications are still discussed controversially. Lesions in the uterine cervix may be classified according to the WHO, the CIN system (Richart 1990) or other national agreements (Munich II; Soost 1990). These systems follow morphological descriptions of histological and cytological aberrations. The systems of WHO and CIN judge upon histological abnormalities in the lower, middle and upper epithelium. Therefore, the degree of dysplasia is either mild or moderate or severe. Other mucous membranes as of the oral cavity, larynx, pharynx, esophagus, bronchus, vulva (Figure 3 e), and penis show a more complex architecture and cannot be seized in a three-step classification. With these tissues, caution must be exercised not to be forfeited by under-diagnoses, because a malignant transformation might already develop from basal cells of the epithelium (Van der Waal et al 1997). American and British textbooks do discuss the classification of dysplasias of the stomach, but German counterparts avoid this problem. Especially the irregularly shaped crypts in stomach (Figure 1 a and b) and colon escaped from the above tripartite classification of premalignant lesions. For the latter topographies, several particular classification systems were created and remain still in discussion.

While the dysplasia in the squamous epithelium of the uterine cervix became the pioneering model of multistep tumorigenesis, the development of cervical glandular dysplasias in closest vicinity remained
unresolved. Furthermore, the used criteria do not reproducibly discriminate hyperplasia from dysplasia in the glandular tissue. In contrast to a three-step mode, the Bethesda nomenclature (National Cancer Institute 1989, Hudson et al 1990) distinguished only between low grade and high grade lesions in the squamous epithelium of the uterine cervix. This two-step system anticipated our classification of nuclear DNA in any type of dysplasia.

The notion of disturbed tissue architecture, loss of cellular polarity and proliferation activity were a matter of course. Nevertheless, the descriptive concepts about dysplasia were arbitrary, at least in part. They suffered from a subjective momentum and appeared as artificial or didactic aids that do not analyse or explain the biological alterations in the affected cells. Therefore, empirical descriptions must be specified by a general principle of premalignant tumour development that can be derived from rules of the cell cycle.

Approach with molecular markers

Within more than 20 years, antibodies have been raised to find indicator molecules for tumorigenesis. The assessment of such biomarkers (e.g. Gerdes et al 1983, Khaled et al 2000, Sun et al 2003) was correlated with the status of tumours, their malignant potential and progression. There are three specific immunochemical targets seen with light microscopy: the cell membrane, the cytoplasm and the nucleus. According to this route, one followed the docking of molecules to membrane bound receptors, the signalling pathway in the cytoplasm and its effectiveness on the genomic loci with respect to the cell cycle.

DNA synthesis during S phase is most significant for the cell cycle and thus a prerequisite for cell proliferation and tumour growth. Most valuable are, therefore, “proliferation markers” such as Ki67 antigen, topoisomerase II alpha and proliferating cell nuclear antigen (PCNA). Ki67 antigen is detected during late G1, S, G2 of interphase and

Figure 1. Low-grade dysplasia in gastric (a-d) and vulva (e-h) mucosae. Nuclei with aberrant DNA contents characterise lesions in tissues with different morphology. In gastric mucosa, the branching and budding of the crypts with mild epithelial atypia (a, b). Late metaphase with aberrant 6.1 c Feulgen DNA content (c). DNA histogram (d) from 150 Feulgen stained interphase nuclei shows enlarged, 40% fraction at 4 c, and 4 nuclei >5 c. The vulva segment shows a clumsy protrusion with club-shaped rete pegs and loss of cellular polarity (e, f). Late metaphase 4.3 c Feulgen DNA (g). Sample of 150 Feulgen stained interphase nuclei (h) shows enlarged, 31% fraction at 4 c, and only 1 nucleus >5 c. HE stain (a,b; e,f); Feulgen (c and g). The proliferating cell compartments were selected for DNA microphotometry.
also in mitosis. This antigen has been shown being correlated with cellular regenerations as well as tumour development and progression (Muñoz et al. 2003). The ambiguity can be resolved by strong histological analysis and experience. Restrictions are also true of topoisomerase II alpha, PCNA and the variety of cyclins. Minichromosome maintenance (MCM) proteins play also an essential part in DNA replication displacing oligonucleotides annealed to single-stranded DNA. However, MCMs are not present in stoichiometric amounts and only a fraction of them actively participates in cell cycle regulation.

Growth factors from outside the cell and their transmembrane receptors initiate the mitogenic cascade. The exemplary epidermal growth factor receptor (EGFR) is such a transmembrane protein with tyrosine kinase activity. Specific antibodies revealed increased levels of EGFR after malignant transformation of squamous cells. However, their frequency and significance in dysplasias is yet not certified.

Cellular differentiation is understood to indicate the end of normal tissue development with cell cycles completed. Tumour progression in dysplasia depends on proliferation without differentiation. E-cadherin is a representative antigen for epithelia. This cellular adhesion molecule plays an important role in cellular growth, differentiation and maintenance of tissue architecture. The onset of tumorigenesis corrupts the functional tissue integrity, and the reduced or absent expression of E-cadherin truly indicates progression of a lesion.

General inhibitors of cell multiplication are the tumour suppressor genes like WWOX at the common fragile site FRA16D (Matsuyama et al. 2004).
Well known is the p53 gene; its protein arrests cells to effect DNA repair or apoptosis. Inactivation or loss of the p53 may occur by genomic damage, nonsense mutations or viral interactions, and is associated with a variety of tumours. Unfortunately, antibodies to p53 were found reactive only in a fraction of malignant entities and remained even more doubtful in dysplasias (Ried et al 1996). A similar judgment is true for the P21/WAF1 tumour suppressor, the transcription of which requires wild type p53 activity.

A cell suicide program can be activated in normal tissues and in dysplasias. But positive results with markers for apoptosis do not reveal whether regeneration will overcome the neoplasia, and negative results cannot decide whether the defence mechanism does not work or there is no need for.

These and other tumour markers have allowed gathering a host of valuable information and have fostered our knowledge about cell function in tumorigenesis. However, the hope for unambiguous antibodies for the potency towards malignant transformation has never been fulfilled. Even the frequently tested antigen Ki67, does not contribute to differentiate between hyperplasia and dysplasia or cancer. Since the aesthetic method of immunohistochemistry remains descriptive providing semi-quantitative records at protein levels, one has to turn to the cell nucleus, which provides general information about genome integrity or its aneuploidy caused by amplifications and deletions.

**Cell cycle disorders in low-grade dysplasia**

Judging upon hyperplasia and its discrimination from the onset of dysplasia is an uncertain court in routine pathology. Within this scope, we shall focus first on the general agreement about early histological changes in low-grade dysplasia (LGD). Enhanced proliferation in the vicinity of stem cells leads to branching and budding of crypts in gastric mucous membranes or to club-shaped rete pegs in squamous epithelia (Figure 1a, b, e, f). Slide based microphotometry allows to attribute cell-cycle parameters to these processes. While the stem cells...
at the basement membrane possess normal nuclear DNA of 2.0 c, some of the post-replication nuclei in cells committed for proliferation scatter ± 0.5 c to the 4 c-value (Figure 1 d and h). Such aberrations resulted from genomic instability. In contrast to hyperplasia (see below), some unrepaired somatic genomes escape the cascade of cell-cycle checkpoints and enter a pathologic mitosis (Figure 1 c and g). These mitotic failures are the characteristics of tumorigenesis in \textit{statu nascendi}.

The DNA range 3.6-3.8 c (Figure 1 h) demands careful analysis, because three possible interpretations apply to such interphase nuclei. First, a nucleus was measured by chance during its S phase. Second, the nucleus originated from an unbalanced telophase (1.8-1.9 c), and its deficient complement passed a complete S phase being now in G2. Third, the nucleus originated from a healthy telophase (2.0 c), but did not replicate completely. This selectively replicated (under-replicated) nucleus stays likewise in G2 (Figure 2). Instances of selective (endo-) replication have been investigated with normal development in different zoological taxa. Underreplication produces a cell genetically similar to one in which chromatin has been lost by elimination (Swift 1969). The records spark the idea that similar incomplete DNA synthesis might occur with human tissue pathology.

Nuclei containing more than 4.5 c match also several alternatives. First, a nucleus was met by chance during its 2nd S phase, i.e. during DNA endoreplication. Second, it originated from an unbalanced anaphase, of which the oversized hemisphere (> 2 c) passed a complete S phase being now in its mitotic G2. Third, the smaller telophase product (< 2 c) has passed two consecutive S phases (endoreplications) within a persistent nuclear envelope. Fourth, a nucleus subject to consecutive endoreplication ceased the synthesis of some (highly repetitive) DNA sequences.

**Progression to high-grade dysplasia**

Cell nuclei with abnormal DNA content probably possess a limited chance to differentiate. If differentiation occurs, tumorigenesis is hampered or even stopped in this early stage. However, continuously uncontrolled proliferation and an increase of unbalanced anaphases multiplies aneuploid genomes. This process causes morphological changes and severe functional disorders classified as high-grade dysplasia (HGD). It was shown earlier that aneuploid division figures precede the manifestation of aneuploid interphase nuclei (Steinbeck 1998 a, b). Untimely proliferation and loss of functions may be followed with immunohistochemical markers as discussed above. However, the degree of dysplasia can readily be measured through the number of pathologic mitoses and of aneuploid interphase nuclei. The frequency distribution pattern of nuclear DNA in HGD shifts apart the LGD pattern (Figure 3: examples from stomach and vulval), resembling a severe aneuploidy known otherwise from malignant tumours.

Similarly, specimens from uterine cervix, skin, oral mucosa and colon mucosa were classified according to morphological criteria and analysed by slide-based microphotometry. Nuclear divisions were measured in 15 µm sections, while 8 µm were sufficient for interphase nuclei. Both sample types were Feulgen stained. The critical parameters were the 4.5 c exceeding rate of mitoses and the 5.0 c exceeding rate of interphase nuclei. The results showed that high and low borders of HGD were tissue specific. These data can be used for discriminations between hyperplasia and LGD and HGD in cases showing doubtful morphology. The data did not contradict the supposition that the occurrence of mitotic failures is a progressive process in dysplasias and beyond, i.e. in malignancies (Steinbeck 1998 b).

The potential malignancy depends decisively on the pool of stem cells and committed cells that might enter asymmetric division. Furthermore, the daughter cells containing aneuploid nuclei have to survive successfully. Success means that the cellular defects, aneuploidy included, shall not be detected by defence mechanisms and shall not be eliminated by apoptosis. Therefore, the pool of dividable but aneuploid cells represents the pool of potential malignancy. In contrast, nuclei subject to complete or incomplete endoreplication can increase aneuploidy, but are comparably harmless. Since endoreplicated nuclei were deviated from proliferation, they do not enhance tumorigenesis. Examples can be found in lesions like atypical adenoma in the thyroid, atypical lipoma (unpublished) or dysplastic nevi (Steinbeck et al 1996).

**Discriminating dysplasia from hyperplasia**

Regarding nuclear DNA contents as measures for genomic integrity, one never finds irregularities in hyperplasia. The histology of such epithelia appears being normal and shows inside-outside polarity. An induced cellular proliferation and maturation occurs
in the right order and at the correct location. Proliferation is under stringent genetic control, because some 90% interphase nuclei possess 2.0 c DNA, and about 10% are in G2 with 4.0 c content. Few nuclei in S phase can be measured only in larger samples. Possible DNA damage will be successfully repaired. Therefore, the observed nuclear divisions are true mitoses coming up with equal telophases at 2.0 c level. The general validity of these rules was examined in cases of inflamed gastric and oral mucosae (Figure 4). The interphase nuclei and divisions obeyed strictly the mitotic rules. Since regeneration is under save genetic control, hyperplasia is a benign and reversible process.

If a histological pattern resembles low-grade dysplasia (Figure 1), only a genomic overview by microphotometry can achieve a discrimination and avoid either under- or over-diagnosis. Therefore, several coinages are inappropriate. Neoplastic or atypical hyperplasia does express uncertainty about the type of lesion seen in the microscope. Records of nuclear DNA contents shall discriminate these cases either belonging to the category hyperplasia or to the category dysplasia.

**Maintenance of high-grade dysplasia**

Proliferation activity in tumorigenesis is characterized by pathologic mitoses and especially by unbalanced telophases, of which the number is increased in HGD. The proliferating aneuploid cells represent the growing proper pool of potentially forthcoming malignancy. Thus, the DNA contents of interphase nuclei display significant scatter about 5 c, and the frequency pattern may resemble that in cancer. Many these nuclei have not been able to rule cellular differentiation, because their surrounding cytoplasm appeared tight, and E-cadherine was not detected in the cell membranes.

A fraction of the larger nuclei might have left the mitotic cycle and switched to DNA endoreplication. This is a dead end for cellular proliferation and therefore without malignant risk. Nevertheless, these nuclei enhance the microscopic pattern of HGD.

The quantitative details about aneuploidy foster the idea that the chromosomal mutations, which bring about the dysplastic phenotype, affect mainly the (somatic) stem cells and the subsequent generation of committed cells in a given tissue. Indeed, the progressive decrease of truly mitotic nuclei was determined within the basal cell layer of the uterine cervix from LGD to HGD and cervical cancer (Steinbeck 1997 b, there Figure 10). Since aneuploidy is a substantial nuclear defect, the status of a dysplastic cell is irreversible. However, even in HGD, transformation and clonal selection have not yet evoked an autonomously proliferating cell line. A dysplastic lesion, therefore, can regress as long as 2 c nuclei are present in healthy stem cells.

Clonal selection works already in dysplasia, when a healthy cell transforms into a tumour cell. Such nuclear transformations occur with random incidence in LGD, but with increased frequency in HGD. This statement found support from comparative genomic hybridization (CGH) that cannot detect specific chromosomal alterations in LGD, but make such accumulated mutations apparent in HGD.

Finally, the discrimination of HGD from carcinoma in situ (Cis) is sometimes controversially discussed. Cis is known from lesions in the uterine cervix, when stem cells are used up, and the superficial cells do not maturate. This pathology might present a lesion restricted to the uterine cervix and cannot be strictly defined for other topographies.

**Pathologic mitoses reflect cell-cycle disorders**

Healthy tissues depend on persistent genomic stability, which is guaranteed by equal bipolar segregation of accurately reduplicated chromosomes. The correct mitosis requires that failures do not occur or become detected and effectively repaired during the cell cycle. Otherwise the affected cells should commit suicide or should be eliminated by defence mechanisms.

The entry into DNA synthesis and the whole S phase appears much endangered by wrong composition and malfunction of the relevant enzyme complexes. Defects of any gene product involved in DNA replication or in chromosome structure is able to cause aneuploid results. However, not only this segment of the cell cycle is protected, but checkpoints have been identified being effective up to the late anaphase (Yang et al., 1997). These findings imply that even an ongoing, but unbalanced distribution of chromosomes might be detected and the forthcoming daughter cells destroyed.

The cascade of multiple checkpoints should exert a tight control over the entire cell cycle. However, dysplasia originates from undetected nuclear defects. Two different modes of control slippage are proposed:

(i) The complete cascade is somewhat leaky, and a
few aneuploid nuclei might successfully enter telophase. The gedanken experiment precludes that a single checkpoint might be responsible, because downstream instances would detect such dysplasia creating defects. The remaining possibility appears highly unlikely that the ultimate checkpoint (in anaphase) could be the only gate for dysplastic cells.

(ii) Genome instability creates a peculiar chromosome constitution that cannot be detected by the cascade of checkpoints. The shaken nucleus performs an unbalanced anaphase and enters an aneuploid telophase that allows constitutive continuation. This model of chromosome lottery has been proposed by Theodor Boveri (1914).

In any case, each type of error passing the anaphase control must do so repeatedly if a dysplastic phenotype shall be established. One cannot discriminate in the microscope, whether pathologic complements will be stopped at any checkpoint or whether they are just running through the cell cycle, anaphase included. This problem, however, does not cut down the diagnostic rule saying that nuclear defects represent a measure for tumorous lesions and their progression.

**Definition of dysplasia revised**

A general classification of dysplasia could not be derived from descriptive approaches, because such lesions exhibit variable phenotypes in different tissues. It is not a surprise that a decision remained difficult between hyperplasia and an early, but irreversible neoplasia. If dysplasia remains undefined, a patient is endangered by under-diagnosis or over-diagnosis and, in consequence, by under-therapy or over-therapy.

The present definition continues the Hansemann-Boveri hypothesis that was improved by pivotal criteria of the cell cycle. DNA measurements of interphase nuclei do support conventional diagnoses and decide on the grade of dysplasia.

LGD is defined by a small number of proliferating cells, a fraction of which can be identified as pathologic mitoses characterized by their aberrant DNA content (Figure 1 c and d). Afterwards, aneuploidy occurs also in interphase nuclei (Figure 1 d and h). Tumour promoting clonal selection, which prevents differentiation, is still at an early onset, because many cell compartments differentiate despite of aneuploidy in others.

HGD is defined by continuous proliferation of aneuploid cells. The number of pathologic mitoses, especially of unbalanced telophases, is highly increased. Interphase nuclei display also enhanced aneuploidy (Figure 2 d and h), while their capacity for differentiation appears restricted.

The revised definition reflects the kinetics of nuclear disorders in correlation with altered tissue morphology. The grade of dysplasia depends, therefore, on the measure of chromosomal mutations and their multiplication through consecutive pathologic mitoses. Additional mutations may enhance the primary mitotic failure. The potential of malignancy and thus the core of tumorigenesis is correlated with the increase of pathologic mitoses and the persistence of defective daughter cells. The product of cellular proliferation and aneuploidy classifies the progress in tumour development and thus the endangering of the patient.

The acting pathologist has not only to judge upon the pattern, the texture and the diameter of cell nuclei, but has to measure the nuclear DNA to ascertain aneuploidy. Recording the frequency of nuclear divisions alone is insufficient practice, because only their DNA contents decide unequivocally about genomic integrity. Enhanced mitotic activity (obvious proliferation) in the vicinity of aneuploid interphase nuclei is in my experience a strong indication that such divisions suffer from pathologic alterations and shall result in unbalanced daughter nuclei.

The present definition of dysplasia was derived from thousands of diagnoses performed in my laboratory. The research focused onto the topographies of uterine cervix, skin, oral mucosa, stomach and colon mucosa. My experience expands also to other epithelial tissues comprising esophagus, larynx, pharynx, bronchia, breast, vulva and penis. More scientific effort, however, will be necessary to decide upon possible premalignant stages of highly differentiated cancers with few chromosomal aberrations. Tracing tumorigenesis in lesions of soft tissues will demand similar efforts.

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