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Review

Pathogenetic mechanisms of nuclear pleomorphism of tumour cells based on the mutator phenotype theory of carcinogenesis

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Summary. The nuclei of the cells of most solid tumours in histopathologic preparations vary in size, shape and chromatin pattern, both from normal nuclei and from each other. These features have not been explained in terms of conventional concepts of nuclear structure and theories of carcinogenesis.

In recent years, the unfolded chromosomes have been shown to occupy "domains" in the nucleus during interphase, providing a relatively uniform density of fine chromatin fibres throughout the nucleus in the living state. This is in contrast to the appearances of interphase chromatin existing as coarse clumps and fibres (heterochromatin and euchromatin respectively) as are seen in histologic preparations. Additionally, the binding of chromatin to nuclear membrane, the possible existence of a nuclear matrix, the functions of nuclear pores, and the attachments of cytoskeletal structures to the outer nuclear membrane are now recognised.

Studies of genetic instability of cancer cells (many random mutations are present in the genome, which vary from nucleus-to-nucleus in individual tumours) have shown that this phenomenon occurs early in tumour formation, can be present in morphologically-normal cells adjacent to tumours, and can result in thousands of genomic events per tumour cell. These observations form the basis for the mutator phenotype/clonal selection theory of carcinogenesis, which proposes that genetic instability is an essential early part of carcinogenesis. Genetic instability has been used to explain significant cell-to-cell variability of behaviour (tumour cell heterogeneity) among cells of individual tumours.

This paper proposes that a high incidence of nucleus-to-nucleus-variable mutation of the genes for factors controlling nuclear morphology in tumours can explain nucleus-to-nucleus variations of histopathologic appearance of these nuclei when some additional effects of histological processing are taken into account.

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Introduction

Appearances of normal nuclei and effects of histopathologic processing

Nuclei are most frequently considered as bags containing randomly tangled, partly unfolded chromosomes, together with ribonucleic acids and histone- and non-histone-proteins (Weiss, 1983; Campbell et al., 1999). In ordinary histologic preparations, coarse clumps and fibres of chromatin are seen, and are often referred to as "heterochomatin" and "euchromatin" respectively (Frost, 1997). However, nuclei of living cells examined by ordinary light microscopy or dark-field microscopy have long been known to show little internal structure (Maximow and Bloom, 1948; Le Gros Clark, 1965). By phase-contrast microscopy, vague fibres (“large scale chromatin fibres”, Sadoni et al., 2000) have been reported, but no other features are seen in most cell preparations. Micromanipulation experiments with fine needles inserted into living nuclei have shown that nucleoli can be moved apparently freely in nuclei, with little apparent distortion of nuclear outline (Mirsky and Osawa, 1961). These results suggest that no large rigid clumps of chromatin (to correspond to histologic "heterochromatin") exist in vivo.

Further support for a relatively uniform distribution of chromatin in living interphase nuclei comes from comparisons of morphology according to histologic processing technique. Nuclei of cells which are air-dried and then fixed with methanol show little internal structure (Bancroft and Stevens, 1996). However, when a shrinkage-promoting fixative such as ethanol is applied while the living tissue is wet (Bancroft and Stevens, 1996; Baker, 1958), a rim of chromatin (“chromatinic rim" – Frost, 1997) and sharply defined central clumps

of chromatin are identifiable. For example, in air-dried, methanol-fixed cells (as in the May-Grunwald-Giemsa method; Fig. 1A), little internal clumping of chromatin is seen. However, in ethanol-fixed "wet preparations" of cells (as in the Papanicolau method, Fig. 1B) sharp chromatin patterns are observed.

Histological processing also involves a series of washing steps, which leaches up to 30% of DNA from nuclei (Bancroft and Stevens, 1996). Remaining nucleic acid in histological sections is probably retained by its pre-fixation binding to fixative-precipitable proteins, especially histones (Baker, 1958).

Appearances of tumour cell nuclei

Tumour cell nuclei in histopathologic preparations often vary in size, in shape, in coloration after staining ("chromatism") and in chromatin pattern (Walter and Talbot, 1996; Frost, 1997; Rubin and Farber, 1999) from normal nuclei, and from each other (Figs. 2-6). Tumour cells also often show abnormal mitoses (Hiem and Mitelman, 1995). These features are sufficiently unique to form much of the basis of the diagnosis of tumours in histopathology and cytopathology. More severe degrees of these nuclear abnormalities often correlate loosely with greater clinical aggressiveness of tumours (Walter and Talbot, 1996; Rubin and Farber, 1999).

There has been little discussion of possible pathogenetic mechanisms of these nuclear abnormalities according to theories of carcinogenesis. The nuclear abnormalities are too marked in many types of tumours to be consistent with a simple excess of any ordinary tissue- or cellular process, such as tissue "differentiation" (specialisation; Del Buono and Wright, 1995), embryological process, or reparative response (Willis, 1948; Iversen, 1995), without at least the action of some additional process. Similarly, the changes are too variable from cell to cell to be explained by a small number only of mutations which are proposed to account for tumourigenesis as "oligo-hit" or "multistage" mutational hypotheses of malignancy (Ilyas et al., 1999; Compagni and Christofori, 2000). This is because the small number of accumulated mutations affect behaviour of all daughter cells equally, and these cells, if secondarily morphologically abnormal, should be morphophologically abnormal in the same way, rather than pleomorphic (Willis, 1948; Iversen, 1995).

Newer concepts of nuclear structure

Chromatin compaction in interphase nuclei, and aggregation during injury and apoptosis

Interphase chromatin is compacted to approximately 10-20% of the degree of compaction of chromosomes in metaphase (Alberts et al., 2002), and is controlled, so that relevant parts of chromosomes may become more unfolded during "activation" of resting cells (for example from "memory" lymphocyte to "activated" lymphocyte). The mechanism of this physiological compaction of chromatin in interphase is unknown (Nicolini et al., 1997; Bradbury, 1998; Qumsiyeh, 1999).

Normal interphase chromatin has long been known to be liable to non-specific collapse ("condensation" "aggregation" or "clumping") in adverse conditions, during which it becomes sticky (Mirsky and Osawa, 1961). This injury-induced chromatin aggregation is usually associated with irreversible inactivation of the chromatin, and loss of viability of the cell (Mirsky and Osawa, 1961). An essentially similar phenomenon has been described as part of apoptosis (Milas et al., 1994; Tang and Porter, 1996; Robertson et al., 2000). Foe and Alberts (1985) showed that reversible chromatin condensation occurs to the nuclear periphery in response to anoxic injury.

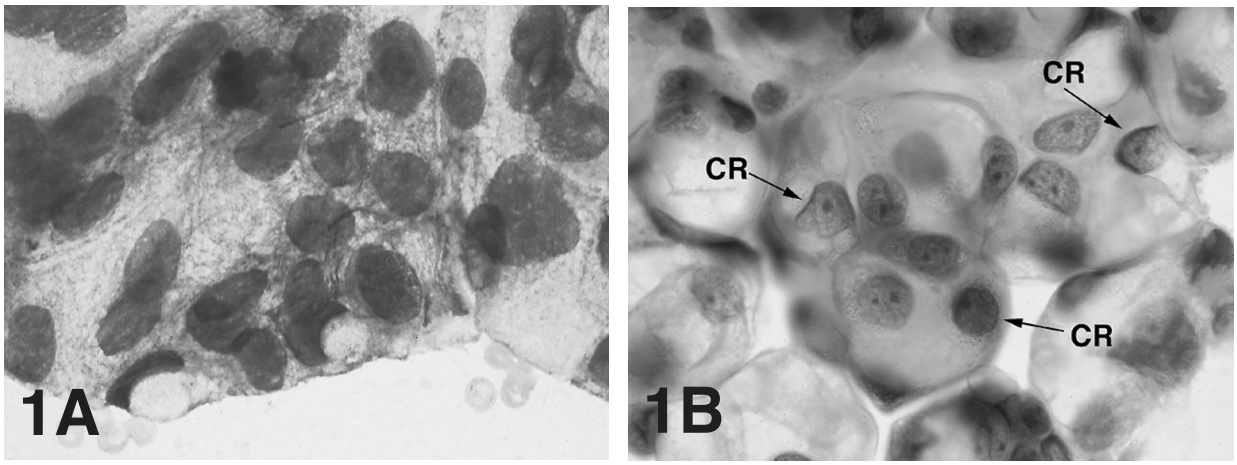

Fig. 1. Variable nuclear chromatin pattern according to method of histological fixation. **A.** Indistinct pattern associated with air-drying and methanol fixation (May-Grunwald-Giemsa stain). **B.** Distinct chromatinic rims (CR) and nuclear chromatin pattern (Papanicolaou stain). From the same case of adenocarcinoma in ascitic fluid. x 600

Chromosomal domains and binding of chromatin to nuclear membrane

The existence of specific, mutually exclusive chromosomal regions of occupation (domains) in the nucleus was proposed in the 1960s (Comings, 1980). This view has now been supported by numerous studies showing that, in interphase, the unfolded chromosomes occupy discrete (non-overlapping) domains which collectively fill the whole interior of the nucleus (Spector, 1993; Lewin, 1997; Cremer et al., 2000; Visser et al., 2000). It has been proposed that each chromosome, and consequently its whole domain, is anchored to the inner nuclear membrane by the centromere of the chromosome (Haaf and Schmid, 1991).

Evidence for chromatin binding to inner nuclear membrane is provided by condensations of chromatin which occur inside the nuclear membrane in some non-mammalian living cells (Mirsky and Ozawa, 1961). The Barr body (a condensed X chromosome occurring only in cells of females) is usually bound to the inner aspect of the cell membrane, in a constant relative position in cells according to cell type. More recently, the binding of chromatin to lamins (see below) has provided a biochemical basis for significant chromatin-nuclear membrane binding.

Nuclear matrix and "scaffold"

Nuclei contain non-histone nuclear proteins which are relatively insoluble, appear to have no direct transcription-factor function, and do not bind significantly to DNA (Nelson et al., 1990; Stuurman et al., 1990; Bosman, 1999). A variety of methods of isolating and visualising these proteins in nuclei have

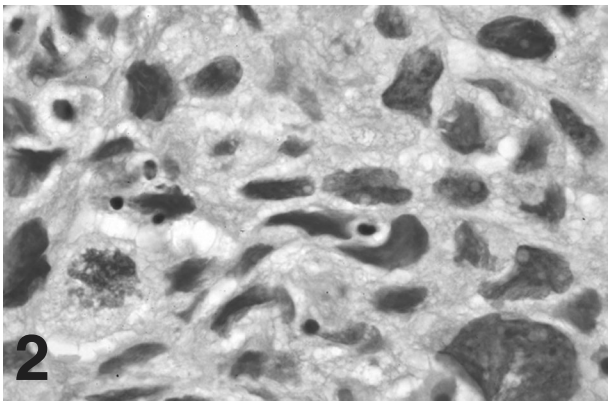

Fig. 2. Nucleus-to-nucleus variation in size and shape in tumour cells. From a case of high grade transitional cell carcinoma of the urinary bladder. H&E. x 600

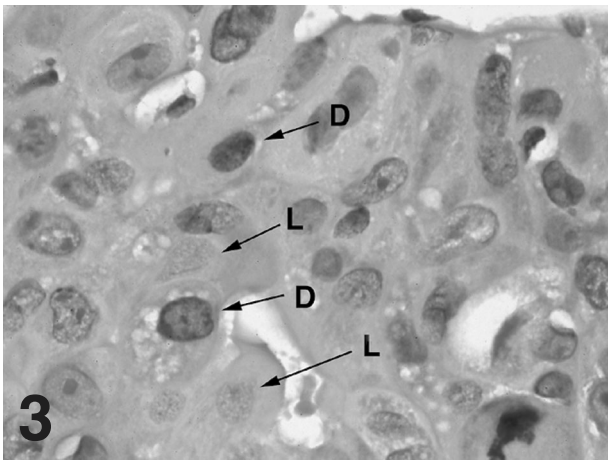

Fig. 3. Nucleus-to-nucleus variation of staining of chromatin by hematoxylin (variable chromatism) in tumour cells. L: light staining; D: dense staining. From a case of carcinoma of the breast. H&E. x 600

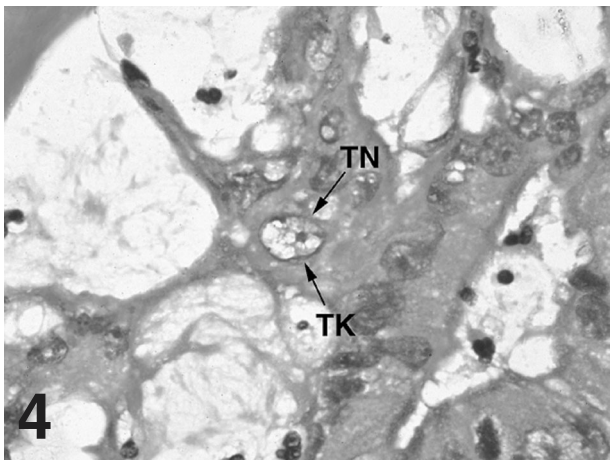

Fig. 4. Nucleus-to-nucleus variation of chromatin pattern in tumour cells. From a case of carcinoma of the breast (not the same case as Fig. 2.). F: fine pattern; C: coarse pattern. H&E. x 600

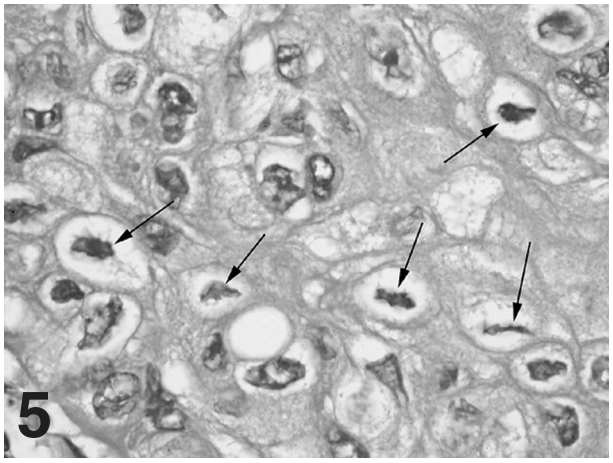

Fig. 5. Variable thickness of the chromatinic rim on the periphery of a single tumour cell nucleus. TN: thin rim; TK: thick rim. From a case of carcinoma of the colon. H&E. x 600

been described (Martelli et al., 2002). These, together with nuclear membrane proteins (especially lamins) and the nucleolar matrix proteins have been suggested to form a nuclear "scaffold" (Haaf and Schmid, 1991) to which transcription-regulating proteins might become attached. It has been suggested that gene transcription may be indirectly controlled by these interactions between transcription factors and matrix proteins (Nelson et al., 1990; Stuuman et al., 1990; Haaf and Schmid, 1991; Bosman, 1999; Martelli et al., 2002). Some doubt has been expressed concerning the existence of a nuclear matrix, as many of the reported findings may be due to preparative artefacts of various types (Pederson, 2000; Martelli et al., 2002).

"Lamins" (Stuurman et al., 1998; Hutchison et al., 2001; Gruenbaum et al., 2000) are one particular family of intranuclear fibrous proteins which are particularly located in the submembranous region of the nucleus ("nuclear lamina"). Lamins are thought to have a structural role, giving the periphery of the nucleus mechanical strength and continuity with the cytoskeleton. Lamins are known to bind chromatin and certain DNA sequences and so may have a role in the control of gene expression.

Nuclear pores

The permeability of the nuclear membrane is limited, selective, and controlled by pores (Bagley et al., 2000; Macara, 2001; Vasu and Forbes, 2001). Molecules up to 20-40kD diffuse passively through pores, while larger molecules, including nuclear proteins, and RNAs require selective mechanisms for import or export. Nuclear pores are complexes of "nucleoporins", of which there are 50-100 different types in vertebrates.

Cytoskeletal-nuclear membrane associations

Micromanipulation with intracellular needles show that the nucleus of many cell types can be moved about in the cytoplasm without apparent detriment to the cell (Mirsky and Ozawa, 1961) suggesting that cytoskeletal-nuclear associations may be weak and/or insignificant. However, most of these studies have been done with cultured cells, and not cells in living, normal tissues, so that subtle functional injuries to the cell might not have been detectable by this method.

In cultured cells, intermediate filaments have special attachments to the nuclear envelope, and radiate from the nucleus to the periphery of the cell (French et al., 1989). Some authors interpret this appearance as indicating a continuous pathway from receptors on the plasma membrane of the cell, through the cytoplasm to the nuclear pores, to serve hormonal responses of cells (Spencer and Davie, 2000). Actin has been identified in nuclei and may have a role in chromatin condensation (Widlak et al., 2002).

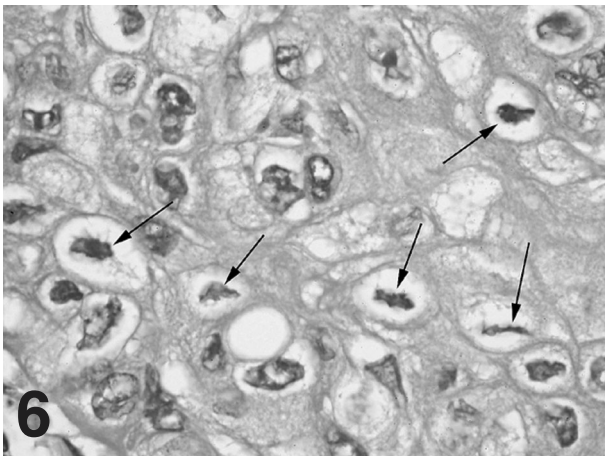

Fig. 6. Nuclei (arrows) shrunken from cytoplasm after histological processing. From a case of carcinoma of the bronchus. H&E. x 600

Genetic instability in cancer cells and the mutator phenotype theory

Beginning in the 1950s, studies of cell lines cultured from primary explants of tumours showed single tumours could give rise to cell lines having markedly different invasive and metastatic properties. This was emphasised by results of studies using athymic mice, in which invasion and metastasis of xenotypic tumours could be studied with ease (Fidler, 1986). The results implied that the proliferative fraction of tumours was not composed of a homogenous population of cells. The term "tumour cell heterogeneity" (Heppner, 1984; Schnipper, 1986; Heppner and Miller, 1998) was used to describe this diversity of cell biological properties of proliferating cells in a single tumour.

Ono (1971) and Nowell (1976) proposed that, in tumours, heterogeneous cell lines could be considered as clones, arising by mutation from precursors, with their survival being dependent on their ability to survive in the particular microenvironment in which they developed. At the time, mutation in tumour cells was thought to be a relatively rare event, and methods to test the possibility of frequent mutation in cancers were lacking.

From the 1980s, molecular biological techniques have advanced to enable these studies to be carried out, and the phenomenon of "genetic instability" in tumours was established (Hill, 1990; Miyagawa, 1998; Tarapore and Fukasawa, 2000). Loeb (Loeb, 1998; Loeb and Loeb, 2000) has used the term "mutator phenotype" for cells showing increased mutation rate, and has proposed that this is essential to the malignant process, as it can provide for the wide diversity of mutations necessary for the growth of tumour cells in the successive microenvironments (local interstitial fluid, lymph, lymph node, blood stream, interstitial fluid of distant organ) which are required for fully developed metastasis to occur (Nicholson, 1984; Fidler, 1997).

Improved methods of detecting mutations have shown much higher numbers of mutations than previously thought likely. Stoler et al. (1999) found that cells of carcinoma of the colon had an average of 11,000

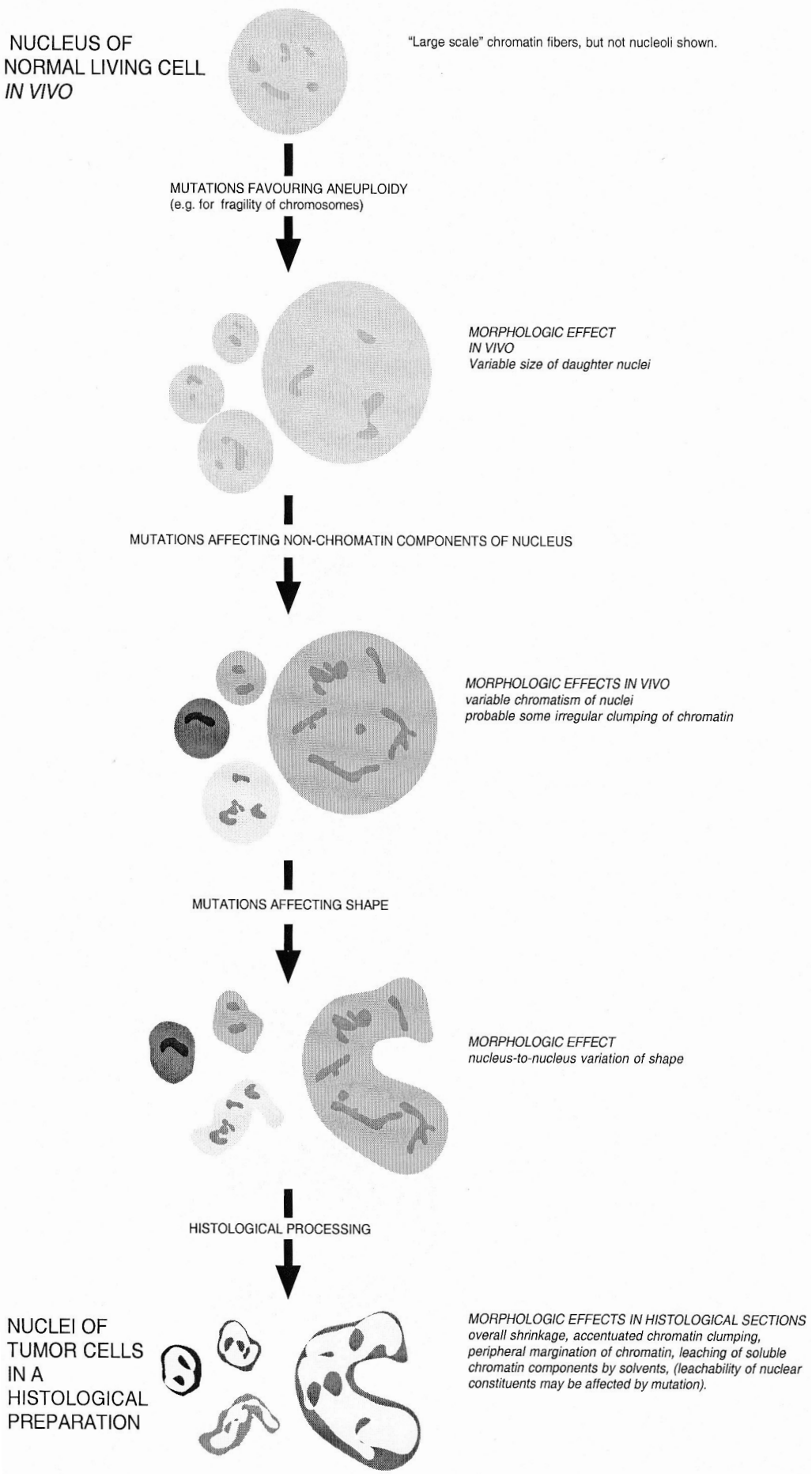

Fig. 7. Diagram of major influences on the appearances of tumour cell nuclei in histological sections. Among tumours, and between cells of the same tumour, the sequence and degree of each effect may vary. The mutator phenotype theory provides a basis for these variations.

"genomic events" per nucleus. Furthermore, mutations are now recognised to be frequent in morphologically normal cells adjacent to tumours (Hittelman, 2001).

A mutational basis for nuclear pleomorphism of cancer cells

If the basic process of malignancy is of a high rate of random mutations occurring randomly in proliferating cell populations, then genes for factors controlling nuclear morphology may well be susceptible to mutation on a nucleus-by-nucleus basis. By taking into account the newer concepts of nuclear structure, and the effects of fixatives and histological processing, a scheme by which the nuclei of tumour cells come to appear as they do in histological sections can be constructed (Fig. 7). In each case, the sequence of events, and intensity of each effect may vary on a nucleus-to-nucleus basis.

Variable nuclear size without alteration of chromatism

This change is most probably due mainly to proliferation and aneuploidy. The latter is probably largely caused by non-disjunction during mitosis (Hiem and Mitelman, 1995).

Variable nuclear chromatism (Fig. 3)

The density of the chromatin in the nucleus can alter by variations of the relative concentrations of chromatin and non-chromatin components. In the context of a histological preparation, several mechanisms might be relevant. Since chromatin once formed can be lost by leaching (Bancroft and Stevens, 1996) (see above), variable susceptibility to leaching of the chromatin in tumour cells may be present. This could occur by way of variable mutation of genes for DNA binding proteins in the nucleus.

In addition, the non-chromatin components of the nucleus may vary. This variation could occur by mutation-induced over- or under expression of these intranuclear components.

Yet another series of mechanisms affecting nuclear chromatism might be dysfunction of nuclear pores (Hood and Silver, 1993, 1999; Sugie et al., 1994). Dysfunctionally increased permeability of nuclear pores might allow non-specific diffusion of cytoplasmic materials into the nucleus. This of itself may variably alter nuclear appearances. Further, abnormal egress of large molecular weight nuclear contents into the cytoplasm might affect RNA transcription and consequently a variety of cytoplasmic functions. Of especial interest in this regard is the recent report by Shekar et al. (2002) that overexpression of the ubiquitin-conjugating enzyme Rad6 in tumour cells leads to nuclear localisation of the protein, multinucleation, centrosome amplification, abnormal mitosis, aneuploidy and transformation.

Dysfunctional decreases of nuclear pore permeability might reduce the flow of transcription factors into the nucleus, and mRNAs out of the nucleus, with consequent interference with the metabolism of the cell.

If any of these abnormalities occurred variably on a nucleus-by-nucleus basis, then nuclear pleomorphism could well be the result.

Abnormal nuclear shape (Fig. 2)

Irregularity of outline of a flexible hollow structure can be due to abnormalities of the internal structures which are bound to the inner surface of the wall, abnormalities of the wall itself of the structure, or abnormalities of structures outside the wall. Tumour cell nuclei could be rendered irregular in shape as follows: First, abnormally short cross-nuclear bands of chromatin or nuclear matrix/scaffold could cause internal angulations of nuclear membrane. Second, aggregated foci of abnormal constituents of the nuclear membrane, could bind chromatin and/or nuclear matrix/scaffold and draw foci of nuclear membrane inwards. This phenomenon could be exaggerated by the use of shrinkage-inducing fixatives, especially ethanol (Bancroft and Stevens, 1996; Frost, 1997). Third, abnormal perinuclear structures, such as intermediate filaments or abnormal residual (post-mitotic) spindle proteins, could form shortened "stricturing" bands around tumour cell nuclei.

Variable mutations could conceivably have a role in variable secondary changes of nuclei, such as "moulding", which implies variable consistency of adjacent nuclei pressed together. The phenomenon of nuclei shrinking away from the surrounding cytoplasm after fixation and histological processing (Fig. 6) could be due to dysfunction of intermediate filaments.

Variable "fineness" and "coarseness" of chromatin pattern (Fig. 5)

Chromatin patterns as identified in formaldehyde-fixed and ethanol-dehydrated histological sections are probably largely a reflection of the coagulability of the chromatin with itself and with non-chromatin components of the nucleus during histological processing (Bancroft and Stevens, 1996). Fine chromatin patterns would imply the presence of intranuclear substances which inhibit the auto-coagulation of chromatin and the co-coagulation of it with other nuclear structures by fixatives. Conversely, coarse chromatin patterns imply the presence of a intranuclear substances which promote chromatin-coagulation and co-coagulation by fixatives. The "intranuclear substances" in both events are likely to be non-histone proteins, which are known to vary in experimental tumours (Forger et al., 1976).

Variable and irregular chromatinic rim (Fig. 5) and the "collapsed chromosome domain nucleus" (Fig. 8)

Irregularity of the chromatinic rim is difficult to

explain in terms of the view of nucleus as an inert bag containing partly unfolded chromosomes (Weiss, 1983) because no mechanism is offered for the chromatin to be especially concentrated in the sub-nuclear membrane region of the nucleus. The most plausible explanation of an irregular chromatinic rim of the nucleus might be that significant chromosome-nuclear membrane attachments do exist, and an abnormally large or small chromosome causes an abnormally large or small segment of the chromatinic rim (Fig. 5).

Fully developed examples of this process can be identified in occasional tumour cell as a nucleus with an empty centre and a rim comprising multiple small nodules of chromatin (Fig. 8). The "chromatinic rim" between the nodules is equally thin between all these structures. The nucleolus is not involved in the process. The nucleus can be large or small. This appearance is consistent with each nodule being a collapsed chromosome according to the chromosomal domain model of nuclear structure.

Conclusion

Nuclear pleomorphism in histological preparations has been identified as a common aspect of the histology of cancer for over 100 years and is universally used for the purposes of diagnostic histopathology and cytopathology. However, a satisfactory pathogenetic explanation for the phenomenon has not been possible in terms of conventional concepts of nuclear structure and common theories of carcinogenesis.

By invoking a high rate of mutation (the mutator phenotype theory of carcinogenesis), to nuclear morphology-affecting structures (nuclear-membrane binding of chromatin, chromosomal domains, the functions of nuclear pores) and taking into account the effects of histological processing on nuclear appearances, satisfactory mechanisms of nuclear pleomorphism emerge.

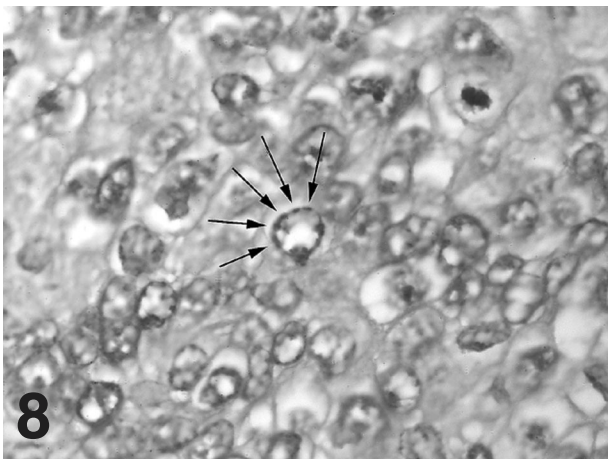

Fig. 8. Tumour cell nucleus showing extreme retraction of chromatin to the inner nuclear membrane, with formation of discrete nodules (arrowed) of chromatin. These nodules are consistent with the collapse of individual chromosomes to the nuclear membrane during histological processing, such that each nodule represents a single collapsed chromosome. Because the focal plane of the micrograph is less than 10% of the thickness of the nucleus, only some of the chromosomes are demonstrated. From a case of carcinoma of the bronchus (the same case as in Fig. 6). H&E. x 600

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