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LIFE PREVAILS OVER DEATH: IS IT GOOD FOR TUMORS?

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ABSTRACT
Mitosis and apoptosis are two processes that oppose each other but are essential for survival of an organism. Homeostasis is achieved when the rate of cell division in the tissue is balanced by cell death. Oral cancer and, in particular squamous cell carcinoma have been repeatedly linked to apoptotic dysregulations. Mounting evidence suggests that oral carcinogenesis is correlated with a progressive accumulation of genetic alterations in molecules that play crucial roles during apoptosis. The relationship between cell growth and cell death in cancer will predict the growth rate of the tumor. Published article on mitosis and apoptosis accessible in the internet are reviewed.

KEYWORDS: Apoptosis, Mitosis, Squamous cell carcinoma.

INTRODUCTION

Life prevailing over death is the most sought after phenomenon. But the victory of life over death in tumors is the most feared one. Control of the cell division cycle is central for governing as when the cell should commit to deoxyribonucleic acid (DNA) synthesis and proliferation versus growth arrest, DNA repair or apoptosis.[1] The accumulation of neoplastic cells can occur through enhanced proliferation, diminished cell turnover, or a combination of both processes. The relationship between cell growth and cell death in cancer will predict the growth rate of the tumor.[2] In the recent past, histological techniques that identify the parameters such as cell proliferation and cell death have become quite significant. Assessment of the number of apoptotic cells and the formulation of the apoptotic to mitotic ratio provides a figure which reflects tumour dynamics even at the light microscopy level and may provide a numerical index of biological behaviour.[3] This review mainly features cell cycle, mitosis, apoptosis and its role in oral squamous cell carcinoma. (OSCC).

Cell cycle

The cell cycle consists of distinct phases. When cells leave a state of quiescence (G0), they enter a first gap phase (G1) before they commit to DNA synthesis (S phase). Many signaling pathways feed into the cell cycle machinery in G1. Also, during this phase, all prerequisites for proper S-phase progression are being checked. Subsequently, a second gap phase (G2) follows, before cells enter mitosis (M), which is the actual cell division phase.[1] After the M phase, each daughter cell may enter Go phase or move on to the G1 phase of a cell cycle. The interphase which is comprised of the G1, S and G2 phases forms the largest part of the cell cycle, but cells in these phases cannot be morphologically recognised. Cells in the mitotic phase can be identified because of the typical appearance of the chromosome sets during the different subphases of the M phase. This has been the basis for mitosis counting on light microscopy, which is the oldest way of assessing proliferation.[4] On the molecular level, the cell cycle is governed by the temporally and spatially fluctuating activities of protein complexes. The core of each complex comprises a cyclin, the essential regulatory subunit and a cyclin-dependent kinase (CDK), the catalytic subunit.[1]

The connection between the cell cycle and cancer is obvious. Cell cycle machinery controls cell proliferation and cancer is a disease of inappropriate cell proliferation. The first genetic alterations shown to contribute to cancer development were gain of function mutations.[5] Dysregulation of cell cycle checkpoint control may lead to independent growth regulating signals. Common causes in cancer include either the aberrant expression of positive regulators, such as cyclins, or the loss of function of negative regulators, such as cyclin dependent kinase inhibitors (CKIs). Loss of function of the tumor suppressor gene product pRb, p53 would remove the brake in the cycling. They appear to be an exclusive target of cyclin D-associated kinases, which is necessary for cell cycle progression.[1] (Figure 1).

1. Mitosis

The actual process by which the cell splits into two new cells is called mitosis.[6] Cell division normally takes place by means of bipolar mitosis which results in the formation of two daughter cells, each with an equal complement of chromosomes derived from the parent cell.[7] The identification and quantification of mitotic cells forms an indivisible part of the histological grading systems used for prognostication of precancerous and cancerous lesions.[8]

1.1 Phases of Mitosis

The segregation of the replicated chromosomes is brought about by a complex cytoskeletal machine with many moving parts called mitotic spindle. The spindle starts to form outside the nucleus while the chromosomes are condensing during prophase (Figure 2A). When the nuclear envelope breaks down at prometaphase, the microtubules of the spindle are able to capture the chromosomes, which eventually become aligned at the spindle equator, forming the metaphase plate (Figure 2B). At anaphase, the sister chromatids abruptly separate and are drawn to opposite poles of the spindle; at about the same time, the spindle elongates, increasing the separation between the poles (Figure 2C). The spindle continues to elongate during telophase, as the chromosomes arriving at the poles are released from the spindle microtubules and the nuclear envelope reforms around them.[6] (Figure 2D).

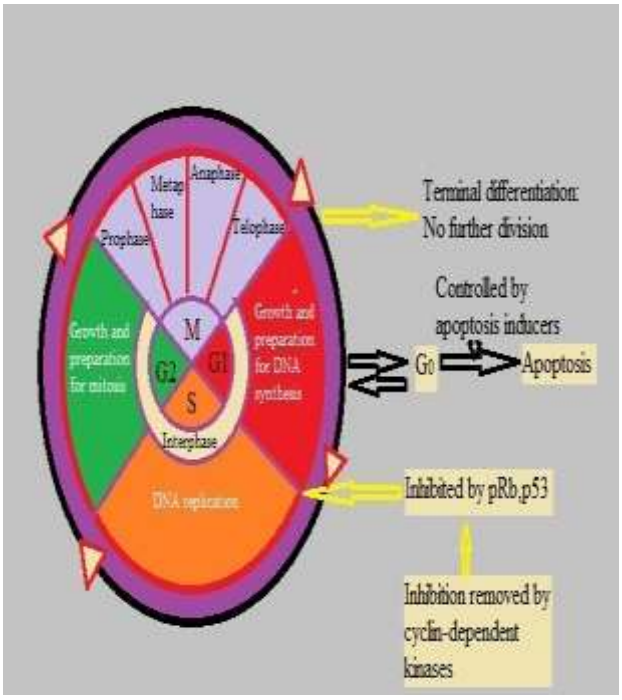

Fig 1: Regulators of Cell Cycle.

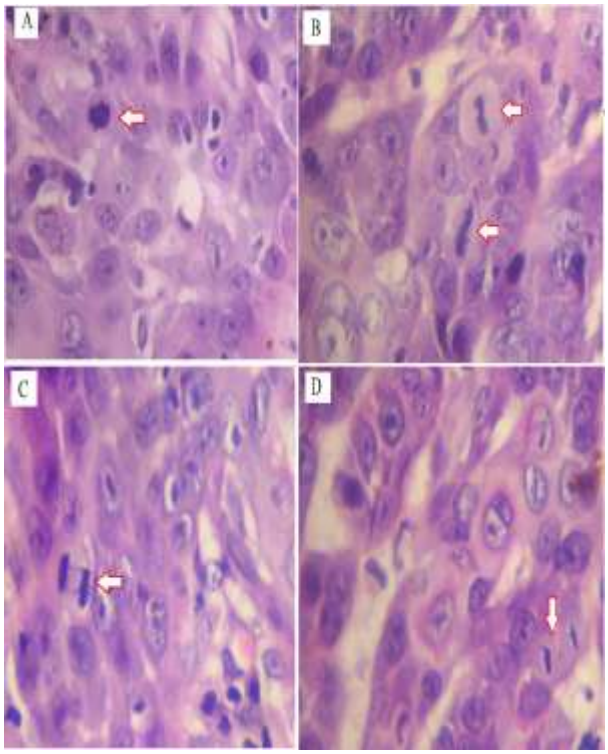

Fig 2: Phases of mitosis. 2A. Prophase. 2B. Metaphase. 2C. Anaphase. 2D. Telophase (H & E stain. x40).

1.2 Methods of Detection-Mitosis

Newer prognosticators like immunohistochemistry, flow cytometry, autoradiography, DNA ploidy measurements are available to determine mitosis but cost and time factors make them less feasible.

1.2.1 Immunohistochemistry

Phospho-histone H3 (PHH3), which is specific mitosis marker, consistently expressed in cells undergoing mitosis, while it is not expressed in interphase cells.[10] Condensation of chromatin during cell division provides protocols for identification and quantification of mitotic cells based on immunohistochemical detection of histone H3 phosphorylated on Ser 10 (H3-P), the critical event occurring during the G(2) to M transition (essential for chromatin condensation), using anti-PHH3, a commercially available antibody.[9] No phosphorylation of histone H3 has been observed during apoptosis, which is also the reason why PHH3 is considered to be a mitosis-specific marker. Other method include assessing proliferative activity using immunomarkers such as MIB-1, AgNOR, PCNA and p53.[10]

1.2.2 Flow cytometry

In this method nuclei in a cell suspension are stained with a fluorescent dye, sucked into the flow cytometer where the fluorescence is excited and measured by means of a photomultiplier system.[4] M-phase cells show lower propidium iodine fluorescence compared to G2-phase cells.[11]

1.2.3 Autoradiography

Incorporation of P^{32} into the DNA of individual cells can be measured by the use of autoradiographs. The period of P^{32} incorporation corresponds to the time of chromosome reproduction and is indicated by X-ray breakage.[12] Incorporated radioactive thymidine can also be visualised by autoradiography and regarded as a gold standard marker of S phase cells.[4]

1.2.4 DNA ploidy measurement

The distribution of a population of cells within the cell cycle generates a pattern known as a histogram and represents DNA ploidy. A DNA histogram is defined as diploid when the predominant or G0/G1 peak is equal to the G0/G1 peak of a known diploid reference cell population and the S and G2M phase contents are relatively low. In normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population forms the G0/G1 peak and 15% of the cells are in the S phase and G2 and M phases.[13]

1.2.5 Stains

A properly stained section and precise use of morphologic criteria are useful in identification of a mitotic figures.[8] Routinely haematoxylin and eosin (H & E) stain is used for estimation of mitotic figures. Toluidine blue, crystal violet, giemsa and feulgen are special stains used to stain nuclear DNA.[14]

The morphologic criteria for mitosis counting proposed by van Diest et al. are as follows.[8,9]

1. The nuclear membrane must be absent indicating that cells have passed the prophase.
2. Clear, hairy extensions of nuclear material (condensed chromosomes) must be present either clotted (beginning metaphase), in a plane (metaphase/anaphase) or in separate clots (telophase)
3. Two parallel, clearly separate chromosome clots to be counted individually as if they are separate.

1.3 Mitosis in OSCC

Cancer and mitosis are closely related. Von Hansemann reported that many of the mitotic divisions in carcinoma did not result in the usual equal distribution of chromosomes to the two daughter cells and also noted many instances of multipolar mitoses. Thus it was concluded that asymmetrical mitosis constituted a definite diagnostic characteristic of carcinoma.[7] The quantification of mitotic figures has been on the backseat over the decades. Counting mitotic figures help in assessing cellular proliferations and aid in histological grading. Thus easy identification and quantification of these figures is an indispensible aid in pathology.[9]

Madhuri R. Ankle et al compared the staining of mitotic cells in haematoxylin and eosin stain with that in crystal violet stain in normal oral mucosa, oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC). Significantly increased mitotic count was observed in crystal violet-stained sections of OED and OSCC respectively, when compared with H & E stained counterparts. No significant results were obtained when comparison of mitotic counts was made between the dysplasia and the carcinoma groups.[8]

Kiran B. Jadhav et al conducted a study where a significant increase in number of MFs was observed in OED and OSCC in comparison with normal oral mucosa.[15]

2. Apoptosis

Apoptosis is a complex, tightly regulated, and active cellular process whereby individual cells are triggered to undergo self-destruction in a manner that will neither injure neighboring cells nor elicit any inflammatory reaction.[16] Apoptosis of cells occurs during normal development and turnover, as well as in a variety of pathological conditions, which includes cancer. In cancer, the balance between proliferation and programmed cell death is disturbed and defects in apoptotic pathways allow cells with genetic abnormalities to survive. Thus, tumor growth is a result not only of uncontrolled proliferation, but also of reduced cell death. A link between the cell cycle and apoptosis is based on several instances in which apoptosis is regulated by genes that are involved in cell cycle progression. There is accumulating evidence that manipulation of the cell cycle may prevent or induce an apoptotic response depending upon the cellular context.[17]

2.1 Mechanisms of apoptosis

Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners. Caspases belong to a family of cysteine proteases that specifically cleave their substrates after aspartic acid.[18] Caspases are classified into initiator caspases 2, 8, 9 and 10, effector or executioner caspases 3, 6 and 7 inflammatory caspases 1, 4, 5 and other cellular caspases includes 11, 12, 13 and 14.[19] There are three pathways by which caspases can be activated. The two commonly described initiation pathways are the intrinsic (or mitochondrial) and extrinsic (or death receptor) pathways of apoptosis. Both pathways eventually lead to a common pathway or the execution phase of apoptosis. A third less well-known initiation pathway is the intrinsic endoplasmic reticulum pathway.[20] (Figure 3) Suppressors of apoptosis are the Inhibitors of Apoptosis (IAP) family proteins.[21]

2.2 Methods of Detection-Apoptosis

There are various techniques to detect and study apoptosis.

2.2.1 Light Microscope

Hematoxylin Staining: With H & E stain, apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin

condensation and this is the most characteristic feature of apoptosis (Figure 4).[22]

Giemsa Staining: Although the borders of the cytoplasm are more accurately distinguished with this method compared to H & E staining, there is no significant superiority.[23]

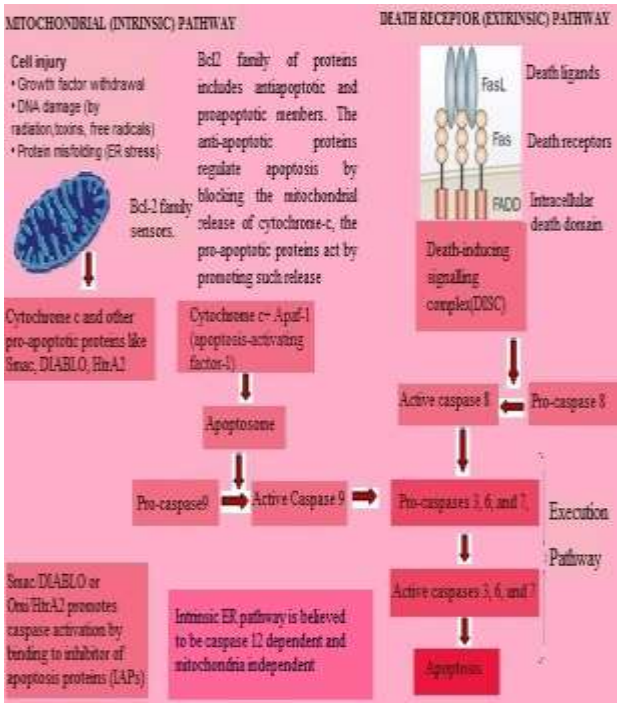

Fig 3: Intrinsic and Extrinsic pathways.

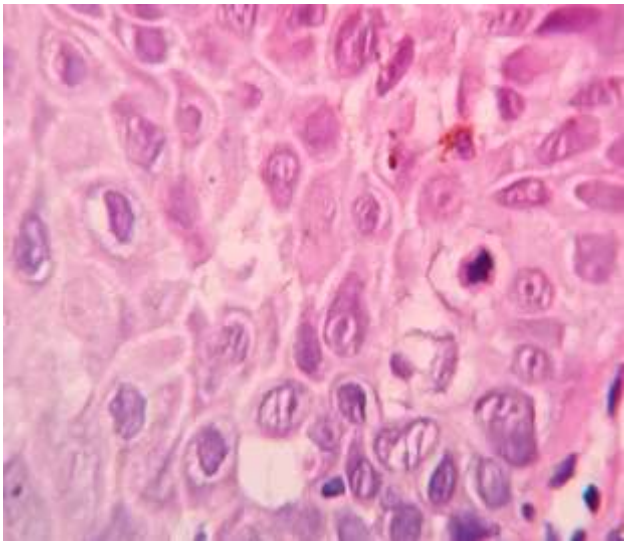

Fig 4: Apoptotic cell (H & E stain. X 100).

2.2.2 Electron Microscope

Defines the subcellular changes better. It shows most conspicuous changes like chromatin condensation phase and electron-dense nuclear material aggregating peripherally under the nuclear membrane; there can also be uniformly dense nuclei. The plethora of information provided is wide in EM.[22]

2.2.3 Immunohistochemistry

Annexin V

At the time of apoptosis the lipid phosphatidylserine translocates from the inner to the outer leaflet of the plasma membrane. Annexin V is a calcium-dependent protein that preferentially binds phosphatidylserine with high affinity. If it is conjugated to a fluorescent tag, Annexin V can be used to detect this early cell surface change of apoptosis.[21]

M30 Antigen Detection

A neoepitope in cytokeratin 18 (CK18), termed M30 neoantigen, becomes available at an early caspase cleavage event during apoptosis of epithelium derived cells and is not detectable in vital or necrotic epithelial cells.[22]

Active Caspase-3 Detection

Procaspase-3 is a protease playing a key role in apoptosis in various different cell types. Procaspase 3 is activated by different stimuli inducing apoptosis and then it is called cleaved or activated caspase 3. The detection of the cleaved form of caspase 3 provides a method that is specific for the determination of apoptosis in the cells/tissues.[23]

2.2.4 Methods to determine DNA changes

Chromatin condensation and DNA fragmentation is one of the apoptotic hallmark. At the late stage of apoptosis, caspase activated endonucleases break the double-stranded DNA. These apoptotic nucleosomal fragments can be resolved by gel electrophoresis as typical DNA ladders. The TUNEL (terminal deoxy-nucleotidyl transferase [TdT] dUTP nick end labelling) assay uses TdT to mark those breakpoints with tagged nucleotides, which are then detected by using enzyme-tagged (for IHC) or fluorescent labelled (for FACS) antibodies.[21]

Apoptotic cells present in normal human tissues and cancers are identified by well-established criteria.[24,25]

(a) Cell shrinkage (smaller in size, eosinophilic cytoplasm with round and smooth margin separating from neighbouring cells).
(b) Chromatin condensation (hyperbasophilic in color and irregular in shape).
(c) Nuclear fragmentation (one or more chromatin pieces, round in shape and variable in size).
(d) Non-inflamed field.

2.3 Apoptosis in OSCC

In SCC, a normal cell transformed into a malignant one due to succession of genetic changes. On the other hand apoptosis is known to eliminate potentially malignant cells, hence reduction of apoptosis can be considered to play a key role in carcinogenesis. Commonly there are three mechanisms by which apoptosis acquire resistance or reduction. These are disruption in balance of proapoptotic and antiapoptotic proteins, reduction in

function in caspases and impaired death receptors signalling.[20]

An increase in the ratio of anti- to proapoptotic Bcl-2 proteins has been detected in various cancers and has been correlated to tumor cell survival and apoptosis resistance.[26]

P53 has been referred to as the 'guardian of the genome' due to its ability to decide the fate of the cell. The cell has a choice to either arrest in G1/S phase and repair the DNA damage or choose apoptosis if the damage is too extensive.[19] Since p53 can promote apoptosis by activating transcription of pro-apoptotic Bcl-2 proteins in the context of DNA damage, nonfunctional p53 can directly be linked to a failure to induce apoptosis after cellular stress.[27]

Inhibitor of apoptosis proteins (IAPs) are a family of proteins characterized by one or more 70-80 amino‑acid baculoviral IAP repeat (BIR) domains. So far, eight human IAP homologues have been identified, among others NAIP, c‑IAP1, c‑IAP2, XIAP and Survivin.[19] The IAPs regulate apoptosis, cytokinesis and signal transduction. IAPs are endogenous inhibitors of caspases. Dysregulated IAP expression has been reported in many cancers.[20]

Mutations within caspase family proteases are not uncommon in malignancies. Caspase-7 proved to be an independent prognosticator and predictor of locoregional recurrence in patients of OSCC.[2] Dysregulation of these enzymes and the pathways in which they are involved can aid in the persistence of mutated cells and promote tumorigenesis.[28]

An immunohistochemical study was conducted by to investigate the gene expression of bcl-2 and bax in OSCCs. In both normal and tumor tissues, the distribution of bax is inversely related to that of bcl-2 and bax plays a role as a dominant inhibitor of bcl-2. When bcl-2 is present in excess, cells are protected and when bax is in excess, cells are susceptible to apoptosis. The ratio of bcl-2/bax mRNA was higher in carcinomas than in the adjacent histologically normal oral epithelium and higher ratios were seen in most of poorly differentiated SCCs.[29]

Ankit Seth et al conducted an endoscopic study, to determine the rate of apoptosis in well, moderately and poorly differentiated carcinoma using H & E stain. An inverse correlation of the apoptotic count/HPF with the histological grade of the tumor was found.[30]

Anshu Jain et al evaluated apoptotic index (AI) to assess the significance of AI as a proliferation marker in premalignant and malignant lesions of the oral cavity using H & E stain. He observed an increased AI as the nature of the lesion changed from oral dysplasia to SCC. High AI in WD SCC as observed by us possibly suggests that tumors that exhibit more apoptosis may be slower growing and therefore may be less biologically aggressive. It has been suggested by some workers that cells with a diminished apoptotic response have an increased propensity for metastatic survival. An association of low AI with higher grade and metastatic phenotype further implies that a low AI suggests a poor prognosis.[31]

Apoptosis is important even when present at low frequency in vivo because of its very short duration. It can be quantified in an attempt to understand the balance of cell proliferation or death in regulated cell numbers. The most universally applicable measuring technique is undoubtedly a fundamental histological assessment based on morphological criteria.[32] Although there appear to be valid biological reasons for a relationship between low apoptotic index and poor prognosis, it is likely that other factors in tumor progression such as mitotic rate and invasive capability also have a confounding influence on tumor behavior.[31]

3. Apoptosis to mitosis ratio

The rate of cell growth must be closely balanced by the rate of cell death. A balance between cell proliferation and cell death maintains cell number homeostasis.

M.A. Birchall et al conducted a study to asses the involvement of apoptosis in the development of oral and oropharyngeal SCC. The results suggest that a change in apoptosis accompanies the onset of invasion in a premalignant lesion of the human oral cavity and oropharynx. However, the mitotic indices are even higher in invasive carcinoma, apoptosis appears to fall markedly. Hence, inhibition of apoptosis may be an important part of the final step from premalignant epithelium to malignancy in vivo.[33]

To explore earlier work, that carcinogenesis is preceded by topographical changes in apoptosis M.Birchall et al conducted a study by increasing sample size. Apoptotic (AI) and mitotic (MI) indices and AI/MI ratio were calculated. MI significantly increased and AI/MI significantly decreased progressing from dysplasia, through CIS, to SCC. However, after inclusion of all variables, only MI remained significant.[34]

AI and MI are parameters that can be assessed easily. An imbalance between apoptosis and mitosis is believed to underlie tumor development. Expression of apoptotic to mitotic index ratio may better reflect the growth potential of the tumor tissue than determination of the mitotic index alone. Quantification of apoptosis and mitosis and determination of apoptotic to mitotic ratio may provide a reliable indication of tumor progression.

CONCLUSION

Mitosis is the process which results in uncontrolled growth of the tumor and apoptosis trys to control it. There is a growing body of evidence that inadequate cell

death as well as excessive cell proliferation contributes to this loss of homeostasis. An insight to apoptosis and mitosis will allow us to develop effective and specific therapeutic approaches.

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