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REVIEW

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Three steps to cancer: how phosphorylation of tubulin, tubulin tyrosine ligase and P-glycoprotein may generate and sustain cancer

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Abstract Transformed cells progress to cancer because they are not eliminated by apoptosis. In this brief minireview I propose, based on published data, that the cell possesses a 'last check point' (LCP) apoptotic step in the form of assembly of nitrotyrosinated α-tubulin onto microtubules. This leads to microtubule dysfunction and ultimately apoptosis. I also propose that cells that escape this LCP apoptotic step develop into cancer. Phosphorylation of tubulin tyrosine ligase (TTL) is postulated to cause escape from LCP apoptosis. Phosphorylation also ensures that cancer cells survive a hostile milieu (e.g. chemotherapy).

Keywords Tubulin modifications · Tubulin tyrosine ligase · Multidrug resistance · Apoptosis · Phosphorylation · Cancer cells

Tubulin, the building block of microtubules, is a heterodimer comprising α and β subunits, each approximately 50 kDa. A third form of tubulin (γ-tubulin) has also been described and is involved in nucleation. The α and β forms of tubulin resolve into a number of isoforms (at least 20 different variants are present in chick brain), which arise partly due to tubulin multigene expression and partly due to a number of post-translational modifications (PTMs, e.g. detyrosination and acetylation of α-tubulin and phosphorylation, polyglutamylation and polyglycylation of both α- and β-tubulin subunits). Most modifications occur in the hypervariable C-terminal region. A number of these modifications (e.g. detyrosination) are unique to tubulin and involve the post-translational addition and/or removal of amino acid(s) to the protein (for review see Idriss 2000a). The precise function of these tubulin PTMs remains largely undetermined. Some are associated with stable populations of microtubules, whilst others seem to influence the binding of motor proteins. Emerging evidence suggests that tubulin modifications may play a role in apoptosis and in multidrug resistance to chemotherapy.

Cells normally undergo apoptosis through activation of biochemical pathways involving caspases (e.g. Alnemri 1997), but other noncaspase-dependent pathways may be at work (see Nicotera 2002). One potentially novel mechanism could be the disruption of the microtubule network through incorporation of nitrotyrosinated α-tubulin. Nitrotyrosine is elevated in abnormal (e.g. cancer and/or infected cells) and is a product of the biochemical reaction between nitric oxide with cellular tyrosine (see Ischiropoulos 1998). The modifying enzyme tubulin tyrosine ligase (TTL) is capable of irreversibly incorporating nitrotyrosine onto α-tubulin which when incorporated into microtubules leads to disruption of the microtubule network, ultimately resulting in apoptosis (see Eiserich et al. 1999) and inhibition of myogenic differentiation (Chang et al. 2002). Tubulin PTMs may influence apoptosis through a caspase-dependent or caspase-independent mechanism. One possible mechanism could be similar to the mechanism proposed for expanded polyglutamines which undergo oligomerization and cause caspase-8 activation in neurodegenerative disorders (Sanchez et al. 2003).

It may also be possible that microtubule 'poisoning' with nitrotyrosine may lead to apoptosis. Microtubule 'poisoning'-induced apoptosis may result through the activity of TTL, which incorporates nitrotyrosine onto α-tubulin. This may define a unique mechanism for the elimination of abnormal cells (e.g. cancer cells) utilizing elevated levels of nitric oxide in these cells and the consequent generation of nitrotyrosinated tubulin in the cells, which causes cell death when incorporated into microtubules. Cells may escape this 'last check point' (LCP) apoptotic route through phosphorylation-induced inactivation of TTL, which I have postulated to

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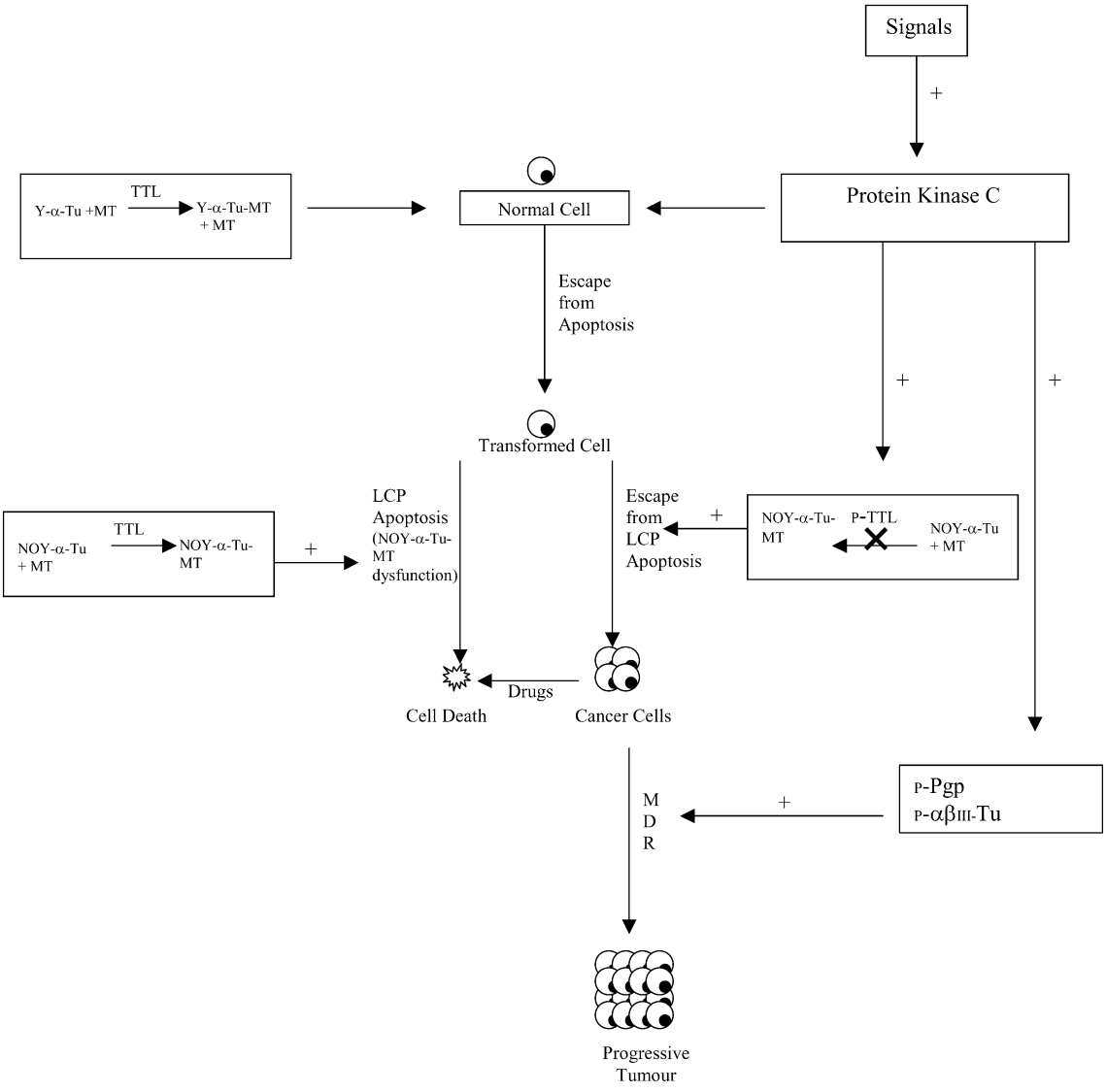

Fig. 1 Three steps in cancer: how phosphorylation of key proteins may cause and sustain cancer. Transformed cells that escape apoptosis are hypothesized to progress to cancer cells proper when they escape last check point (*LCP*) apoptosis. A crucial step in escaping LCP apoptosis is postulated to involve phosphorylation of tubulin tyrosine ligase (*TTL*). Phosphorylated TTL (*p-TTL*) is incapable of incorporating tyrosinated α-tubulin (*Y*-α-Tu) into microtubules (*MT*). It also cannot ligate nitrotyrosinated α-tubulin (*NOY*-α-Tu) to form nitrotyrosinated MTs (*NOY*-α-Tu-MT) so that transformed cells survive solely on MTs consisting of detyrosinated α-tubulin. Cancer cells are maintained by further phosphorylation events allowing them to survive a hostile milieu. Phosphorylation of P-glycoprotein increases (+) the efflux of anticancer drugs out of the cell, whilst phosphorylation of tubulin may interfere with drug binding. Both phenomena possibly contribute to multidrug resistance (*MDR*). Protein kinase C is a likely candidate for such phosphorylation events subsequent to stimulatory signals

be phosphorylated with protein kinase C (PKC) at a serine residue close to its ATP binding site, preventing ATP binding and inactivating the enzyme (Idriss 2000b; 2000c), leading to cancer. Cells that escape nitrotyrosinated α-tubulin-mediated LCP apoptosis are postulated to survive with a population of microtubules consisting of detyrosinated tubulin. The tubulin tyrosination cycle therefore may be under the hierarchical control of phosphorylation and may perturb 'LCP' apoptosis when the system is disturbed (e.g. through kinase activation or phosphatase inactivation as applicable to TTL).

Phosphorylation may further sustain cancer by activating multidrug resistance (MDR) in tumor cells. MDR to chemotherapeutic drugs is a major obstacle in the treatment of cancer in patients. The efflux activity of P-glycoprotein (Pgp) is mainly responsible for this phenomenon, but it may also be due to other novel mechanisms. PTMs, such as phosphorylation, are emerging as potential players in the regulation of MDR in cells. Phosphorylation has been linked to enhanced activity of Pgp (e.g. Idriss et al. 2000a). Direct modification of other cellular proteins in tumor cells may also lead to the phenomenon of MDR. One interesting candidate is tubulin, which is a target site for a number of anticancer drugs (e.g. *Vinca* alkaloids). Although many of the tubulin modifications lie at the C-terminus of

tubulin, they may affect the conformation of the protein close to the drug binding sites, preventing drug binding. The effective concentration of the drug within the cell may then be reduced through the activity of phosphorylated Pgp.

Other modifications such as phosphorylation or acetylation of tubulin may play a more direct role in preventing drug binding since they lie closer to the N-terminus. Unfortunately, no one has as yet investigated drug binding as a function of posttranslational modifications of tubulin or determined which antitubulin drugs induce expression of which tubulin-modifying enzymes. However, initial evidence does correlate tubulin PTMs with MDR. Tubulin detyrosination has been reported in breast cancer and is linked to tumor aggressiveness (Mialhe et al. 2001). However, MDA-MB-231 breast cancer cells contain only tyrosinated tubulin and low level monoglutamylation in certain isoforms (Rao et al. 2001). Additionally, differences have been detected in the in vitro assembly rates between tyrosinated and nontyrosinated α-tubulin isoforms in the presence of Taxol (Banerjee and Kasmala 1998) and a recent study has shown that the levels of tyrosinated tubulin increase in Taxol-resistant MCF-7 breast cancer cells (Banerjee 2002). Tyrosination may act synergistically with other PTMs to influence binding of drugs, such as Taxol to tubulin. The structure of tubulin complexed with Taxol has recently been described (Snyder et al. 2001), but the structure lacks the C-terminal portion or the known PTMs to ascertain if any are involved in regulating drug binding. Such findings are potentially of clinical significance if PTMs at the C-terminus of tubulin interfere with the binding of drugs to tubulin.

In conclusion, the idea that posttranslational modifications of tubulin confer resistance to chemotherapeutic drugs (e.g. *Vinca* alkaloids) is a plausible one and evidence in the literature is starting to emerge in support of this hypothesis. Understanding the full causes of MDR will lead to more effective cancer therapy in the future.

PKC-induced phosphorylation may play three crucial roles in cancer development and maintenance (Fig. 1). These include inhibition of nitrotyrosinated tubulin-induced apoptosis through inhibition of TTL activity, decreasing the effective intracellular concentration of anticancer drugs by activating Pgp through phosphorylation and possibly prevention of drug binding to tubulin through the addition of a phosphate at a site affecting drug binding to tubulin (e.g. at the *Vinca* alkaloid binding site). PKC phosphorylation of tubulin has not been extensively characterized, but PKC δ is known to phosphorylate β-tubulin in vitro (Chen et al. 2002). Recently phosphorylation of β-tubulin with G-protein-coupled receptor kinase 2 has been mapped to Thr409 and Ser420 (Yoshida et al. 2003), in the intermediate domain of the protein molecule. The βIII-tubulin isoform is known to be phosphorylated in brain cells and dephosphorylated with protein phosphatase 2A (Khan and Luduena 1996). Recently $\alpha\beta$III-tubulin has been shown to be less sensitive to Vinblastine-induced inhibition of polymerization than other $\alpha\beta$-tubulin isotypes, although the study did not correlate this observation to the phosphorylation status of βIII-tubulin (Khan and Luduena 2003). Additionally, elevations in the levels of $\alpha\beta$III have been observed in tumors treated with Taxol (Ranganathan et al. 1996).

PKC expression is elevated in cancer cells and selective targeting of specific PKC isoforms may control MDR (O'Brian et al. 2001) and/or apoptosis, for example through regulation of Pgp and TTL activities (e.g. Idriss 2000c; Idriss et al. 2000a, 2000b).

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