TXR1-mediated thrombospondin repression: a novel mechanism of resistance to taxanes?

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The usefulness of chemotherapeutic agents for the clinical treatment of cancer relies on their toxic effects on dividing cells. While most anti-cancer drugs directly affect DNA synthesis or integrity, the vinca-alkaloids and taxanes act by disrupting the cellular microtubule network [depicted schematically in Fig. 1; for review, see Zhou and Giannakakou 2005]. In addition to providing structural integrity as part of the cytoskeleton, microtubules function as the cell’s freeway system by providing the infrastructure along which cargo can be transported by motor proteins such as kinesin and dynein. During cell division, microtubules are critically important for the formation of the mitotic spindle, which dictates the proper segregation of chromosomes during mitosis.

Microtubules are synthesized through the polymerization of α- and β-tubulin subunits on their fast-growing, or plus, ends. At the same time, they are degraded on their slow-growing, or minus, ends by the release of α/β dimers into the cytosol. The combined processes of assembly and disassembly cause microtubules to be in a state of “dynamic instability,” which allows the growing or shortening of individual microtubules as required. Anti-microtubule agents exert their anti-cancer effect by disrupting normal microtubule dynamics in distinct fashions. Whereas vinca-alkaloids are able to bind to free tubulin, thereby preventing microtubule assembly and concomitant mitotic spindle formation, they cannot associate with polymerized tubulin present in microtubules. In contrast, taxanes interact with polymerized tubulin to cause microtubule stabilization. As a result, cells become overloaded with microtubules and are unable to resolve the mitotic spindle, causing a mitotic arrest or apoptosis.

Taxol, the prototype chemotherapeutic of the taxane family, was isolated in the early 1970s from Taxus brevifolia. In recent years, taxol/paclitaxel and taxotere/doxetaxel, a semisynthetic taxane identified in the 1980s, have become part of standard drug treatment regimens and are widely used in the clinic to treat a variety of human malignancies, including breast, ovarian, lung, gastric, and prostate cancers. The latter two are of special interest because taxanes may prove to offer some advances in the treatment of these otherwise refractory diseases.

The cytotoxic effects of taxanes

The direct effects of taxanes on microtubule dynamics, cell-cycle arrest, and apoptosis have been subject of intensive study [Fig. 2]. Although both docetaxel and paclitaxel enhance microtubule stability, in vitro and in vivo studies have revealed important differences between the two drugs [Gligorov and Lotz 2004; Zhao et al. 2005]. For example, docetaxel has a much higher affinity for β-tubulin compared to paclitaxel, resulting in both increased potency and elevated cytotoxicity [Riou et al. 1992]. Furthermore, whereas docetaxel affects centrosome formation in the S-phase of the cell cycle, paclitaxel disrupts mitotic spindle formation in G2/M [Hennequin et al. 1995].

Moreover, depending on drug concentration, dose-schedule, and the intactness of different cell-cycle checkpoints, the outcome of drug treatment can differ substantially. Low concentrations of paclitaxel (<10 nM) generally do not result in a G2/M arrest but instead cause cell death following aberrant mitosis, whereas higher concentrations of the drug (10–100 nM) cause a terminal G2/M arrest [Torres and Horwitz 1998; Chen et al. 2003]. This arrest does not result in apoptosis unless the G2/M checkpoint is disrupted. In the latter case, cells will either apoptose during mitosis upon activation of the spindle checkpoint or following mitotic catastrophe, in which cells progress through an aberrant mitosis after which the cellular default apoptotic pathway is activated [Castedo et al. 2004]. Alternatively, mitotic slippage, in which cells exit from mitosis in the absence of chromosome segregation, can result in the generation of tetraploid cells, which apoptose in the presence of an intact tetraploid checkpoint [Chen et al. 2003]. When this checkpoint is incapacitated, however, mitotic slippage can give rise to aneuploid cells. Finally, very high
concentrations of paclitaxel (>200 nM) cause extensive polymerization of tubulin, leading to the formation of microtubule bundles and necrosis (Yeung et al. 1999). The fact that taxane treatment has proven less beneficial for the treatment of colorectal cancer compared to other solid tumors may be related to the fact that these tumors show a high incidence of chromosomal instability and defects in cell-cycle checkpoints, the presence of which is in essence required for the cytotoxic effects of both docetaxel and paclitaxel (Swanton et al. 2006).

Different downstream events have been implicated in taxane-induced apoptosis, including the phosphorylation and concomitant inactivation of Bcl2, activation of JNK and Raf1, as well as both caspase-dependent and -independent apoptotic mechanisms [Zhao et al. 2005]. In summary, the outcome of taxane treatment appears to be concentration-dependent and cell-type-specific, complicating the elucidation of a single mechanism that underlies the cytotoxic response to drug treatment.

**TXR1 activation represents a novel mechanism of taxane resistance**

As with other anti-cancer drugs, tumor cells can acquire resistance to the cytotoxic effects of taxanes. Increased drug efflux from cells through enhanced expression of the multi-drug-resistance protein P-glycoprotein-1 (PGP1) is the most frequently described mechanism [van Ark-Otte et al. 1998]. Although the clinical benefit of combined therapy with taxanes and PGP1 inhibitors remains to be determined, there is evidence to suggest that the levels of PGP1 expression may be an indicator for the response to treatment (Yeh et al. 2003). Interestingly, changes in β-tubulin isotype expression, with a switch from class I to class III [Ranganathan et al. 1998; Dumontet et al. 2005; Mozzetti et al. 2005; Sève et al. 2005], show a high incidence of chromosomal instability and defects in cell-cycle checkpoints, the presence of which is in essence required for the cytotoxic effects of both docetaxel and paclitaxel (Swanton et al. 2006).

**Figure 1.** Microtubules are instrumental in maintaining cell viability. *(A)* Microtubules, which point with their plus end away from the centrosome from which they originate, have important roles during different phases of the cell cycle. In interphase, the microtubule network provides the cell with a system along which proteins, vesicles, and organelles can be transported by dedicated motor proteins. Kinesin transports cargo toward the plus end, whereas dynein walks along the microtubules toward the minus end of the microtubules. During mitosis, microtubules form the mitotic spindle, which is required for the faithful segregation of sister chromatids to opposite ends of the cell. Only after the spindle checkpoint has been passed and the spindle is properly resolved, cells are able to undergo cytokinesis to give rise to two daughter cells, each containing a diploid set of chromosomes. *(B)* Microtubules are highly dynamic. *(Left)* Microtubules can be conceived as hollow fiber-like structures, which are composed of 13 filaments. Each filament is a tubulin polymer, with alternating α- and β-tubulin subunits. New tubulin subunits are added to the plus end of the microtubule, whereas degradation occurs on the minus end. The regulation of both assembly and disassembly ensures proper microtubule dynamics. *(Middle)* Microtubule-targeting anti-cancer drugs affect the microtubule network in different ways. Vinca-alkaloids bind to β-tubulin monomers, thereby preventing them from being incorporated in growing microtubules. The balance between assembly and disassembly is distorted, and the cellular microtubule network collapses, causing mitotic failure. *(Right)* Taxanes, such as paclitaxel and docetaxel, have the opposite effect. Instead of preventing microtubule assembly, these drugs interact with β-tubulin that has already been incorporated into microtubules. They stabilize the structure and prevent disassembly on the minus end. As a result, cells become overloaded with microtubules, again causing a mitotic crisis.
have also been shown to occur upon taxane exposure, in vitro as well as in tumors from patients receiving chemotherapy. In fact, multiple studies have reported changes in isotype expression to be predictive for the outcome of taxane treatment. Mutations in the α- and β-tubulin genes (Giannakakou et al. 1997; He et al. 2001; Achiwa et al. 2003; Martello et al. 2003; Wang et al. 2005, 2006; Hari et al. 2006) have also been reported, although their influence on the clinical response to taxane treatment is less well established (Monzó et al. 1999; Maeno et al. 2003; Mesquita et al. 2005).

In the current issue of Genes & Development, Lih and coworkers (Lih et al. 2006) describe a new mechanism of taxane resistance involving the novel protein TXR1, which implicates an important role for the anti-angiogenic and proapoptotic protein thrombospondin (Fig. 3).

Using a modified gene-trap vector (Li and Cohen 1996), the investigators set out to search for genes that conferred resistance to paclitaxel. To this end, human prostate cancer cells infected with the gene-trap virus were grown in the presence of 10 nM paclitaxel for 2 wk, after which a single resistant clone, 18, which showed ~2.5-fold reduced sensitivity to paclitaxel, was chosen for further study.

Insertion of the gene-trap vector results in expression of an antisense transcript driven by a tetracycline-regulatable promoter, which can be expected to inhibit the expression of cellular genes in the region. Indeed, in the presence of tetracycline, sensitivity of clone 18 cells to both paclitaxel and docetaxel was restored to wild type, indicating that mere integration of the gene-trap vector had not been the cause of the observed resistance. Additional analysis showed that the gene-trap virus had landed in the 5'UTR of a novel gene, the expression of which was—quite unexpectedly and through an unknown mechanism—up-regulated about five times in cells with the insertion compared to parental cells. The investigators named the novel gene, previously identified as DKFZp566J157, TXR1, for taxol-resistance gene 1.

TXR1 encodes a small protein without any known functional domains or protein motifs. It is composed of proline residues for up to 30% and harbors a long stretch of serine residues in the C-terminal tail. In order to link TXR1 more directly to taxane resistance, the investigators used siRNA vectors directed against TXR1 to show that the reduction of TXR1 levels back to wild type in clone 18 cells restored sensitivity to paclitaxel. In addition, a reduction in TXR1 levels in the parental cells increased paclitaxel sensitivity, indicating that endogenous TXR1 may protect cells from taxane cytotoxicity.

To directly show that TXR1 conferred taxane resistance, the investigators generated cells expressing exogenous TXR1 from the same tetracycline regulatable promoter as the one present in the gene trap virus, and showed that these cells indeed displayed increased resistance to paclitaxel. As the overall intracellular levels of paclitaxel were comparable in parental and clone 18 cells, drug efflux mechanisms such as PGP1 overexpression were dismissed as a possible mode of TXR1-mediated resistance. In addition, α- and β-tubulin expression as well as microtubule dynamics appeared to be affected to a similar extent in clone 18 and parental cells in response to paclitaxel. Both of these observations suggested that a different, more downstream event likely contributed to taxane resistance upon TXR1 activation.

An unexpected role for TSP1 in taxane-induced apoptosis

To hunt down the mechanism behind TXR1 mediated resistance to paclitaxel, Lih et al. (2006) compared microarray expression profiles of parental cells and cells harboring an activated TXR1 gene. While TXR1 itself was the most up-regulated outlier, the most prominent down-regulated gene in this analysis was thrombospondin-1 [TSP1]. This inverse correlation between TXR1 and TSP1 expression was retained upon the addition of tetracycline to the cells: As TXR1 expression in clone 18 cells was reduced, the cells showed a concomitant increase in TSP1 levels back to wild type. Lih et al. were further able to show that the regulation of TSP1 by TXR1 occurred at the transcriptional level, since a luciferase reporter construct containing the TSP1 promoter was activated following tetracycline-mediated down-regulation of TXR1 expression.
TSP1 sparked immediate interest because of its previously reported anti-angiogenic and proapoptotic activities in oncogenesis [Sid et al. 2004]. In order to directly demonstrate that TSP1 mediates taxane sensitivity downstream of TXR1, the investigators administered increasing concentrations of recombinant TSP1 to clone 18 cells, thereby restoring the sensitivity to paclitaxel in these cells. In addition, after treating clone 18 cells with a combination of TSP1 and 10 nM paclitaxel, there was an increase in the number of apoptotic cells compared to treatment with either TSP1 or paclitaxel alone. Importantly, the observed effect was specific for taxanes, since the addition of TSP1 left the cells’ sensitivity to other anti-cancer drugs unaffected. Thus, TSP1 may be an effector of the apoptotic response to taxane treatment and repression of TSP1 following up-regulation of TXR1 may represent a novel mechanism of taxane resistance.

The finding that TSP1 is involved in taxane cytotoxicity is in agreement with a previous observation by [Yoo et al. 2002], who showed that docetaxel treatment of head and neck squamous cell carcinoma cell lines resulted in the induction of TSP-1. So how does TSP1, a secreted, extracellular matrix protein, directly affect cell killing by taxanes? And moreover, how can the cytotoxic effects of TSP1 be so specific to this class of anti-cancer drugs?

In a first attempt to elucidate the biological mechanism behind paclitaxel induced cell-killing through TSP1, Lih et al. (2006) directed their attention to two cell surface receptors known to mediate the TSP1 signal, CD36 and CD47, also known as integrin-associated protein (IAP). Since only the latter was expressed on their cells, they hypothesized that TSP1 was most likely to act via CD47. Indeed, when three different TSP1 mimic peptides were added to their cells, only 4N1K, which binds to CD47, but not the mutated peptide 4NGG or ABT-510, which binds to CD36, decreased cell survival in the presence of 10 nM paclitaxel. Conversely, inhibition of CD47 signaling by use of an anti-CD47 antibody increased cell viability in the presence of paclitaxel.

Implications for chemotherapeutic intervention?

Given the diverse effects of taxanes at different stages of the cell cycle depending on drug, dose, and cellular makeup [Fig. 2], it will be important to determine whether TSP1 is a general effector of taxane-induced cytotoxicity.

Lih et al. (2006) showed that TXR1 confers resistance to low concentrations of docetaxel and paclitaxel. As discussed above, under these circumstances, taxanes are thought to cause aberrant mitosis, which will lead to apoptosis in the presence of an intact p53-checkpoint. Since TSP1 is a known p53-target, it will be interesting to see whether TXR1 counteracts p53-mediated TSP1 activation in the presence of taxanes.

With performing their gene-trap screen by growing human prostate cancer cells in the presence of 10 nM paclitaxel for a prolonged period of time, Lih et al., in essence, used an experimental drug treatment schedule that is reminiscent of what has become known as metronomic or low-dose chemotherapy in the clinic [Kerbel and Kamen 2004].

Chemotherapeutic regimens generally comprise the intermittent treatment with high drug doses, which not only requires prolonged intervals between cycles of treatment due to toxic side effects but which might also cause tumor cells to acquire [multidrug] resistance. In contrast, the more frequent or even continuous low-dose administration of the same chemotherapeutic agents has shown to inhibit tumor angiogenesis by targeting the nonmalignant endothelial cells of the growing tumor vasculature [Browder et al. 2000]. The promise of metronomic chemotherapy is underscored by reports that tumors that have stopped responding to high-dose treatment schedules might, in some cases, still be sensitive to the same chemotherapeutic agents when these are administered in a low-dose/high-frequency schedule [Kerbel and Kamen 2004].

Metronomic chemotherapy exerts its anti-angiogenic effects by causing the up-regulation of endogenous angiogenesis inhibitors. Interestingly, one of the best-studied targets in this regard is TSP1 [Bocci et al. 2003]. Of note, TSP1 levels are elevated following low-dose treatment with a range of different cytotoxic agents in addition to taxanes, suggesting that a general stress- or damage-signaling pathway results in increased TSP1 expression. The TSP1 protein contains a number of functional domains, each of which mediates one of its very diverse functions [Fig. 3B]. These include binding sites for heparin sulphate proteoglycans, CD36 and CD47. In addition, TSP1 may also directly interact with integrins.

Much of what we know about the role of TSP1 in oncogenesis relates to its anti-angiogenic effects mediated by endothelial cell killing. There is ample evidence showing that, in contrast to the induction of apoptosis in tumor cells involving CD47, as described here by Lih et al. (2006), TSP1 induces endothelial cell death through CD36 (Asch et al. 1987; Dawson et al. 1997; Guo et al. 1997). This eventually results in caspase-dependent apoptosis involving p38 and JNK [Jimenez et al. 2000, 2001]. A synthetic peptide, ABT-510, which is able to interact with CD36, is therefore able to mimic these anti-angiogenic activities. Both in vitro and in vivo, ABT-510 has been shown to promote CD95-mediated endothelial cell killing [Quesada et al. 2005; Yap et al. 2005]. In addition, ABT-510 has been shown to synergize with cisplatin and cyclophosphamide in endothelial cell killing. Phase I clinical trials using the peptide are underway [Hoekstra et al. 2005, 2006]. The observations made by Lih et al. suggest that it might be worthwhile to invest in similar studies using the 4N1K peptide, which is able to interact with CD47.

Although Lih et al. (2006) did not investigate the mechanism of cell killing downstream of CD47, TSP1-mediated CD47 activation has previously been shown to result in caspase-independent apoptosis in hematopoietic cells [Mateo et al. 1999, 2002]. However, signaling through CD47 was recently also reported to augment Fas/CD95-mediated apoptosis [Manna et al. 2005].
which proceeds through a classical, caspase-dependent pathway. In addition, CD47 mediated cell killing has also been described to proceed via a novel mechanism, which is independent of caspase and cytochrome c activation but which requires cAMP activation via heterotrimeric G-proteins [Manna and Frazier 2003]. The same activity might mediate the effects of TSP1 described by Lih and colleagues, as they are able to block the apoptotic response by treating the cells with pertussis toxin. Interestingly, TSP1 was recently reported to mediate caspase-independent apoptosis through CD47 in leukaemia cells in vitro, indicating that this might be a more general phenomenon in cancer cells [Saumet et al. 2005].

At this point, Lih et al. (2006) only provide in vitro data showing the importance of TSP1 for the induction of the apoptotic effect of taxanes. It is difficult, if not impossible, to extrapolate any of their findings to an in vivo situation. A recently published in vivo study describing the low-dose chemotherapeutic treatment of rats grafted with a prostatic adenocarcinoma, described the induction of TSP1 following treatment with a low dose of paclitaxel. In spite of this, no reduction in tumor weight was observed, whereas this was the case when the rats received cyclophosphamide [Damber et al. 2005]. To complicate matters further, similar to the dose-dependent effects of taxanes, different doses of TSP1 have been shown to have opposing effects on cell proliferation [Isenberg et al. 2005]. Thus, TSP1 is likely to have diverse effects on different cell types, depending on the presence of cell surface receptors as well as on TSP1 levels.

Clearly, it will be necessary to take a closer look at the different activities of TSP1. With regard to oncogenesis, both the anti-angiogenic effects on endothelial cells via CD36 and the proapoptotic effects on tumor cells via CD47 may have important clinical implications. If future studies support the results presented in the current study, hallmarking TSP1 as an important mediator of taxane-induced apoptosis, it might be worthwhile to explore adjuvant therapy with different TSP1 mimetic peptides as a possible way to increase the effectiveness of taxane treatment, thus not only preventing blood vessel formation but also inducing apoptosis of malignant cells. This might even allow a reduction in the doses of docetaxel or paclitaxel that are administered, thereby reducing adverse side effects such as peripheral neuropathy in addition to improving clinical outcome.

Concluding remarks

As paclitaxel and docetaxel have become part of standard chemotherapeutic regimens in the clinic, taxane resistance has become a frequently encountered phenomenon. In addition, the introduction of novel anti-microtubule agents, such as epothilones, into the model of what is currently known about the effects of taxanes on microtubule dynamics and mitosis? How important are the different cell cycle checkpoints for their activities? And finally, given that paclitaxel is most often used in combination with other chemotherapeutic drugs, especially with cisplatin, it would be interesting to determine whether TXR1 can also confer resistance to a combination of these agents?

Future studies will also have to be directed at unraveling the mechanism by which TXR1 causes TSP1 repression. Given its primary amino acid structure, the investigators propose a role for TXR1 in reducing TSP1 expression through interaction with known TSP1 regulators. Although this is an attractive hypothesis, it remains to be demonstrated. It also raises the question of whether TXR1 may have a more general role in repressing transcription. Interestingly, in an attempt to generate cells overexpressing TXR1, Lih et al. (2006) were unable to obtain clones expressing TXR1 from a CMV-driven cassette. This may indicate that cells are very sensitive to the levels of TXR1 and only tolerate a very modest increase in TXR1 protein expression. This raises the question of whether high-level overexpression of TXR1 can cause cell killing or cell cycle arrest by itself, as well as whether TSP1 is involved in this. In addition to determining the precise biological roles of TXR1, it will be worthwhile to investigate whether TXR1 is overexpressed in human tumors that have acquired chemotherapy resistance. In any case, the data presented by Lih and coworkers tell us that we should look beyond the anti-angiogenic effects of TSP1 when it comes to treatment with taxanes, since there appears to be an unexpected role for TSP1 and CD47 in mediating paclitaxel sensitivity.

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