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Commentary

Microtubule-based Motor Proteins: New Targets for Enhancing Drug Delivery?

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INTRODUCTION

With the development of new protein, peptide, and oligonucleotide drugs, a major challenge in the area of drug delivery is facilitating the uptake and retention of these polar compounds. An obvious strategy for enhancing uptake into the target tissue is by utilizing the endogenous cellular transport machinery involved in membrane traffic. Although previous attempts at facilitating drug delivery by coupling drugs to ligands which are taken up via receptor-mediated endocytosis have been successful, little work has attempted to directly stimulate the target proteins which mediate membrane traffic to further enhance drug uptake and retention. This commentary highlights our current understanding of a major component of membrane traffic, microtubule (MT)[2]-based vesicle transport. The purpose of this commentary is to summarize the recent progress on the function and regulation of MT-based transport with particular emphasis on the involvement of the MT-based motor proteins, cytoplasmic dynein and kinesin, that are responsible for driving vesicle movements along the MT path. Since these motor proteins have potential as new targets for enhancing drug delivery, future directions are outlined with emphasis on areas of direct benefit to drug delivery research.

Routes of Entry for Polar Drugs via Membrane Traffic Pathways

Membrane traffic pathways mediate the movement of membrane-bound components between the plasma membrane and defined intracellular membrane compartments including both the endocytic (early and late endosomes, lysosomes) and biosynthetic/secretory pathways (endoplasmic reticulum, Golgi, trans-Golgi network) (Figure 1). Since endocytic vesicles form by invagination of plasma membrane, a major route of entry into cells for macromolecular drugs is by endocytosis. Extracellular components including polar drugs can gain entry into the endocytic pathway in soluble form (fluid-phase endocytosis), through adsorption to plasma membrane that is subsequently internalized (adsorptive endocytosis), and by binding to plasma membrane receptors that subsequently undergo internalization (receptor-mediated endocytosis). Both fluid and membrane components may be recycled to the plasma membrane from endosomal compartments within a short time frame or routed in a longer recycling pathway which parallels the biosynthetic/secretory pathway via the Golgi and trans-Golgi network. If not recycled, internalized components may move to the lysosomes for degradation. In cells polarized into apical and basolateral domains (epithelia and hepatocytes), a further layer of complexity must be considered, since membranes can not only be internalized and recycled as described but can also be transported from apical to basolateral or from basolateral to apical plasma membranes in a process known as transcytosis.

Understanding how membrane traffic is both up- and down-regulated is critical for drug delivery. Reports of carrier-mediated gene and oligonucleotide delivery by coupling to ligands such as asialoglycoproteins and transferrin, which are internalized through receptor-mediated endocytosis, are examples of drug delivery strategies which have utilized endogenous transport machinery (1–8). Since the molecular events involved in endocytosis, sorting and recycling are imperfectly understood, strategies which currently use the endocytic machinery to enhance uptake and increase specificity can certainly be improved as additional information about regulation of the targets within membrane traffic pathways becomes available. In the target tissue for a particular drug, a desirable goal would be to increase its intracellular accumulation. The same endpoint could be achieved in several ways including stimulation of endocytosis, alteration of sorting within endosomes such that the percentage of internalized drug sorted to late endosomal and lysosomal pathways rather than recycled to the plasma membrane is increased, and finally, inhibition of recycling of endosomal components.

Role of Cellular Microtubules in Membrane Trafficking

MTs are cytoskeletal polymers consisting of α and β tubulin. The αβ tubulin dimers are assembled in a head to tail array into protofilaments; 13 protofilaments are in turn organized around a central hollow core to form the MT (9, 10). Assembly of tubulin into MTs is a highly regulated process mediated by intracellular calcium, GTP, MT-associated proteins, and other factors (9, 10). As constituents of the mitotic spindle, MTs play a major role in dividing cells; however, the interphase

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[2] **ABBREVIATIONS:** MT, microtubule; KHC, kinesin heavy chain; KLC, kinesin light chain; DHC, dynein heavy chain; DIC, dynein intermediate chain; DLC, dynein light chain.

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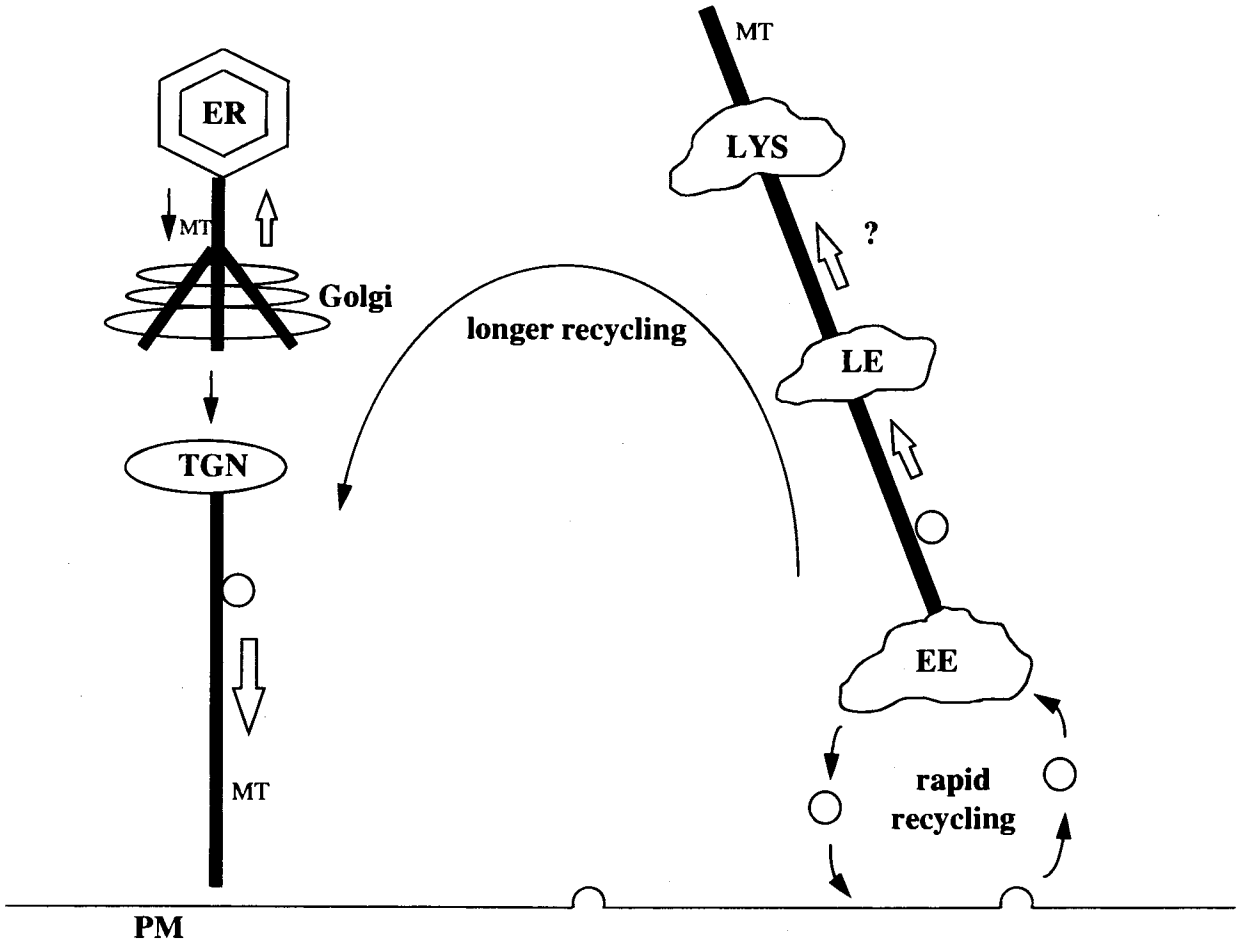

Fig. 1. Membrane traffic in an unpolarized cell. Endocytic traffic involves the uptake of extracellular components from the plasma membrane (PM) to the early endosomes (EE). This uptake can be receptor-mediated or occur non-specifically through fluid-phase or adsorptive endocytosis. From the early endosome, some soluble and membrane components are recycled while others are further internalized to late endosomal (LE) and lysosomal (LYS) compartments in a MT-dependent pathway. Many drugs internalized in soluble or ligand-bound form are destined for LE/LYS compartments where ideally they can diffuse across the compartmental membrane before they are degraded in the acidic, protease rich environment of the lysosomes. Recycling of components from the early endosome can also take place in a longer pathway via the Golgi and trans-Golgi network (TGN). This later recycling pathway converges at the Golgi and/or TGN with the biosynthetic pathway which originates with the synthesis of membrane proteins in the endoplasmic reticulum (ER) which are then transported to the Golgi and TGN. Dark heavy bars indicate MTs. Hollow arrows indicate steps known to involve MT-dependent vesicle transport.

MT network also plays an essential role in interphase and differentiated cells. The head-to-tail organization of asymmetric tubulin dimers into protofilaments provides the MT with biochemically distinct ends, which are referred to as plus (fast-growing) and minus (slow-growing). In unpolarized cells like fibroblasts, the minus-ends are clustered in the perinuclear region at the MT-organizing center (MTOC) and the plus-ends radiate out to the cell periphery (11, 12). This simple model is outlined in Figure 2. MT polarity and organization can therefore provide asymmetry in an otherwise symmetrical environment.

As outlined schematically in Figure 1, MTs provide an intracellular scaffold which facilitates membrane traffic events by supporting the movement of membrane vesicles between membrane compartments. As outlined in detail in several recent reviews (11–13), substantial evidence supports a role for MT-dependent vesicle movements as components of the late endocytic and secretory pathways, and more recently in the recycling of membrane from the Golgi to endoplasmic reticulum. Although internalization of receptor-ligand complexes into endosomal vesicles and the vesicular transport of these complexes to early endosomes is widely assumed to be MT-independent, some recent data also suggests that MTs may play a role in receptor internalization at the plasma membrane (14, 15). In addition to facilitating vesicular transport, the MT network anchors membrane compartments such as the endoplasmic reticulum and Golgi apparatus in their unique morphologies and in their specific cellular locales. In sum, this evidence suggests that MTs play a major role in interphase membrane trafficking events.

Two Cytoplasmic Motors Have Been Identified

Two MT-dependent motor proteins have been identified as candidates for the intracellular motors powering MT-dependent

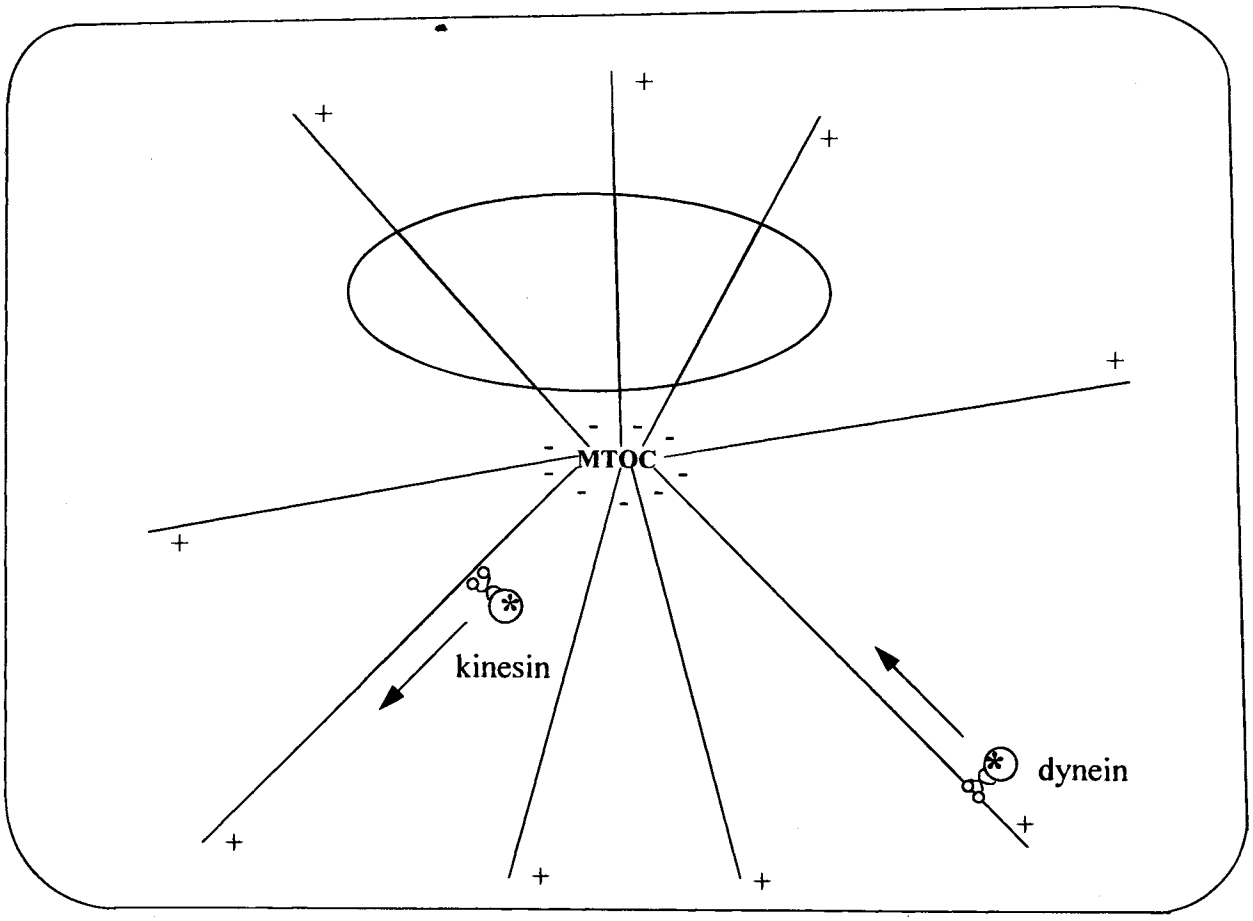

Fig. 2. MT organization in an unpolarized cell. MTs are organized with minus-ends anchored at a central perinuclear organizing center called the MT-organizing center (MTOC). MT polarity directs cytoplasmic MT-based vesicle transport driven by the motor proteins kinesin and cytoplasmic dynein: kinesin is a MT plus-end directed motor while cytoplasmic dynein is a MT minus-end directed motor.

vesicle transport; kinesin, a MT plus-end directed motor and cytoplasmic dynein, a MT minus-end directed motor (16) (Figure 2). These proteins use ATP energy to generate the mechanochemical force necessary for vesicle translocation along the MT path. Accessory proteins may also be required for vesicle motility (11, 17, 18).

Kinesin from a number of sources is composed of 110–135 kD heavy chains (KHC) and 60–80 kD light chains (KLC) organized as a heterotetramer with 2 KHCs and 2 KLCs (16, 19). The KHC NH_2-terminal head domain is termed the motor domain and is the site of MT binding and nucleotide hydrolysis. The head domain is connected by a long flexible rod domain to a short carboxy-terminal tail which is the region of association with the light chains and also with the target membranes.

It has been proposed that the KLCs are predominantly involved in the binding of the motor to its "cargo". Kinesin binding to target organelles is thought to be receptor-mediated; one receptor, kinectin, has recently been identified (summarized in review 12) although additional receptors may exist. As yet unidentified accessory proteins are also known to facilitate kinesin-driven vesicle motility (17). Since the original identification of kinesin, it has become clear through genetic and functional analysis that there are many variations on this original "prototype" cytoplasmic motor. The kinesin superfamily of proteins now includes interphase plus-end directed organelle-based motors as well as mitotic kinesins that facilitate chromosomal and mitotic spindle movements and that in some cases promote MT minus-end movements (19).

Cytoplasmic dynein is a multisubunit complex consisting of two heavy chains (DHC, >400 kD each), three to four intermediate chains (DIC, 70–74 kD) and four light chains (DLC, 40–60 kD) (16, 20). The DHCs, which contain the nucleotide binding sites, have globular heads which form the sites of interaction with the MT. The DICs and DLCs have been proposed to mediate the motor association with membrane cargo and/or to regulate dynein activity. Also an important mediator of cytoplasmic dynein function is the dynactin complex, which was originally identified as an accessory factor of unknown composition that stimulated cytoplasmic dynein-based vesicle motility in vitro (18). Subsequently, the dynactin complex has been shown to contain several polypeptides including dynactin (21), an actin-related protein (22, 23) and other polypeptides of unknown function.

The progress in our understanding of MT-based motor function and regulation has followed a major technological advance in microscopy; this achievement was the use of video-enhanced differential interference contrast microscopy to image MTs (25 nm diameter) and small vesicles in vitro such that

several assays could be developed for biochemical measurement of MT-based motor activity (24, reviewed in 25). These assays are diagrammed in Figure 3. One simple measurement of in vitro motor activity is the MT gliding assay. In the absence of membranes and in the presence of MTs and nucleotides, motors adsorbed to the surface of a glass coverslip can promote the movement of MTs along the surface of the glass. Since the minus- and plus-ends of the MT can not be resolved by video-enhanced differential interference contrast microscopy, MT gliding can not reveal the directionality preference (minus- or plus-end directed) of the motor. However, purified axonemes nucleate MT assembly at the minus-ends; the polarity of MTs polymerized from axonemes is therefore apparent by video microscopy. MTs polymerized from axonemes can be used to determine motor directionality: if the axoneme (MT minus-end) leads the activity is a plus-end directed motor, and if the MT plus-end leads the activity is a minus-end directed motor (26). Video microscopy can also be used for vesicle motility assays which reconstitute in vitro the nucleotide-dependent movement of crude or purified membranes along MTs or axonemes attached to the glass coverslip (17). Continued advances in the understanding of the regulation of these MT-based motors will certainly continue to require the use of these in vitro assays.

MT-dependent Motor Proteins Facilitate Membrane Traffic

Cytoplasmic dynein and kinesin were originally identified and characterized from neuronal tissues, and these motors have subsequently been shown to be integral components of fast axonal transport of vesicles in the retrograde (cytoplasmic dynein) and anterograde (kinesin) directions (11). Further studies have identified a cytoplasmic role for these proteins in a variety of other eukaryotic cells. A simple prediction based on the organization of MT minus and plus ends in unpolarized cells would be that inward-directed or retrograde MT-dependent movements would be driven by cytoplasmic dynein and outward-directed or anterograde MT-dependent movements would be driven by kinesin (Figure 2). In some cases, a clear role for an individual motor protein consistent with this prediction is indicated by experimental studies. For instance, several studies support a role for cytoplasmic dynein but not kinesin in receptor-mediated endocytosis, specifically the sorting and transport of ligand-containing vesicles from early to later endocytic compartments (27–29). Likewise, kinesin has been implicated as the motor promoting the movement of secretory granules to the plasma membrane in T-cells (30).

However, identification of clear roles for each of the MT-dependent motor proteins in traffic has been more difficult in other instances because of an overlapping distribution of the two motors across cellular membranes. Membrane organization of compartments anchored on MTs may involve both MT-dependent motors. Cellular maintenance of tubulovesicular membrane compartments like the lysosomal network (31) and endoplasmic reticulum (32) has implicated kinesin; however cytoplasmic dynein is also associated with lysosomal membranes (33) and can mediate endoplasmic reticulum formation in vitro (34). Although kinesin is enriched in purified Golgi membranes isolated from liver (35), functional studies also implicate cytoplasmic dynein in formation and maintenance of the Golgi apparatus (36).

Clearly, some overlap in the distribution of MT-based motors exists on cellular membranes, with both kinesin and cytoplasmic dynein associated with anchorage of membrane compartments involved in endosomal and secretory/biosynthetic pathways. The roles of the motors on each of these membranes may be direct, i.e., as the engine which drives movement from one compartment to another. Alternatively, there may be instances in which kinesin works in dynamic opposition to dynein-mediated movement in the resting state, and the dynamic is shifted in favor of one motor or the other

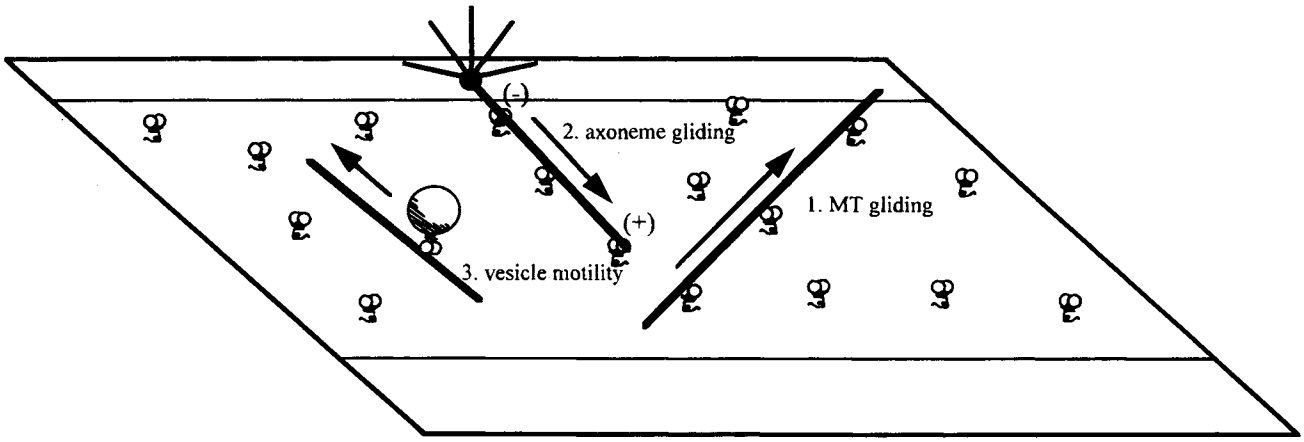

Fig. 3. In vitro assays for MT-based motor proteins. By using video-enhanced differential interference contrast microscopy, the nucleotide-dependent movement of MTs (1) and axonemes (2) can be detected. Although MT-gliding does not reveal the polarity preference of the motor, this preference can be discerned by performing gliding assays with the axoneme which has detectable minus- and plus-ends. Vesicle motility (3) is the assay which most closely reconstitutes in vitro the physiological process of MT-based vesicle transport. Vesicle motility assays can be used not only to probe the activity and regulation of MT-dependent motors but also the requirement and regulation of accessory factors and membrane receptors in MT-based vesicle motility. In the vesicle motility assay, MTs attached to the coverslip with polylysine or by centrifugation serve as a substratum for the nucleotide-dependent movement of membrane vesicles driven by MT-based motor proteins. Each of these in vitro assays allow the measurement of several parameters of MT-based motor activity including frequency of movement (MTs, axonemes, or vesicles), velocity (MTs, axonemes, or vesicles), and run length (vesicles only).

in the stimulated state. Finally, in organelles dependent upon MT-based motors for anchorage on MTs, continuous activity by both motors may be required to maintain morphology and membrane flow to and from the organelle. Each of these dynamics may be important to consider from a drug delivery standpoint.

Motor Protein Regulation

Both kinesin and cytoplasmic dynein have multiple phosphorylation sites and constitute major targets of regulation for membrane traffic. Regulation of MT-based motors may mediate several motor properties, including MT binding, nucleotide hydrolysis, and membrane association. Kinesin regulation has recently been reviewed (37). Briefly, multiple phosphoisoforms of KHC and KLC exist in neurons, with higher levels of kinesin phosphorylation correlated with increased membrane binding (38). However, the nature of the signal transduction pathways involved in kinesin regulation are as yet unclear. Treatment of metabolically labeled neurons with agents affecting cAMP-dependent and Ca^{2+}-dependent pathways failed to elicit major changes in phosphorylation, with the exception of the reduction in KLC phosphorylation by Ca^{2+}-ionophore (39). In vitro, phosphorylation of neuronal kinesin by cAMP-dependent protein kinase caused reduced affinity for synaptic vesicles (40). Recently, correlation of kinesin phosphorylation in vitro and in vivo revealed that activation of both cAMP-dependent protein kinase and protein kinase C increased KLC phosphorylation, but at different sites (41). In this same study, kinesin binding to calmodulin in vitro could be partly blocked by prior phosphorylation with cAMP-dependent protein kinase. We have demonstrated stimulation of several parameters of MT-dependent vesicle movement in intact cells by okadaic acid, an inhibitor of protein phosphatases 1 and 2A, which is correlated with the finding of okadaic acid-induced kinesin phosphorylation and activation in vitro (42, 43). Our recent studies also indicate that isolated kinesin activity from acinar cells is stimulated by prior treatment of the cells with carbachol, a cholinergic agonist that activates protein kinase C-dependent pathways (Hamm-Alvarez, da Costa, Yang, Wei, Gierow and Mircheff, manuscript submitted).

The distribution of cytoplasmic dynein has been shown to be responsive to agents that alter protein phosphorylation. In cultured fibroblasts, cytoplasmic dynein normally localized to lysosomes (33) can be dispersed to a diffuse cytoplasmic pool by serum-deprivation or calcium (44). Additionally, the serine-threonine protein phosphatase inhibitor, okadaic acid, can mimic the effects of serum deprivation (45). Labeling studies have revealed that the okadaic acid-induced redistribution of cytoplasmic dynein is accompanied by increased phosphorylation of DHC (45). In neurons, a recent comparison of the extent of cytoplasmic dynein phosphorylation in the total cellular pool versus the population derived from anterograde-moving vesicles has revealed that DHC phosphorylation in the cellular pool is more extensive (46); since the anterograde-moving vesicles are thought to be driven by kinesin, associated cytoplasmic dynein would be presumed to be in an inactive state in transit to the synapse where it could engage in retrograde transport. This study suggests a positive correlation between DHC phosphorylation and activity, although the mechanisms of regulation have not been identified.

FUTURE DIRECTIONS

Although activation of cytoplasmic dynein and kinesin appears to be an obvious way to enrich the cellular uptake and retention of polar drugs, many questions regarding the regulation of these cytoplasmic motor proteins and their accessory proteins and receptors remain unanswered at this point. Clearly, additional information about the mechanisms of regulation, including the identity of the protein kinases and phosphatases involved, is necessary. Also, little information about the relationship between changes in cytoplasmic dynein and kinesin activity that occur in response to agents which are known to accelerate fluid-phase or receptor-mediated endocytosis is available, although this information is clearly essential for utilizing these motors as targets for improved macromolecular drug delivery.

Although stimulation of an individual motor activity may be associated with certain unidirectional membrane traffic events (i.e., kinesin-driven movement of secretory granules following stimulation with secretagogue), it seems logical that the opposing motor might be coordinately regulated or have its activity altered within a short time frame in order to maintain membrane compartments at a steady state. Lending support to this speculation of coordinate regulation of MT-based motors, previous work on the regulation of intracellular MT-dependent vesicle movements has shown that although changes in frequency of vesicle movements over a 20-fold range could be elicited in CV-1 cells by different pharmacological agents, the ratio of inward to outward movements remained constant at steady state (42). If enhanced uptake could be achieved by a transient stimulation of MT-based motor activity, coordinate regulation of inward and outward MT-dependent vesicle transport might not be a major impediment to drug delivery. For instance, if the diffusion rate of drug from endosomal compartments to the cytoplasm is sufficiently rapid, the increased total amount of this drug which could enter a cell over time through increases in inward and outward MT-based vesicle transport could facilitate increased cytoplasmic concentrations of drug.

Another major question particularly relevant to the area of drug delivery is whether motor activation could promote short term changes in intracellular sorting pathways. For instance, if cytoplasmic dynein activity were increased, could additional drug normally internalized to endosomal components be sorted to lysosomes? In this situation, the prolonged intracellular retention time of internalized drug due to traffic of the drug further into and out of the cell could allow additional drug to diffuse from the endosomal pathway into the cytoplasm. This could occur even if the recycling traffic were coordinately increased.

An example of a situation in which increases in MT-based motor activity could enhance delivery and retention of a polar drug is illustrated in the hypothetical scheme in Figure 4. Cytoplasmic dynein has been implicated in the centripetal movement of components from early to late endosomal compartments (27–29). Specific enhancement of cytoplasmic dynein activity could promote additional vesicular transport of drug which would normally be immediately recycled from early endosomal pathways (Figure 4A) to late endosomal and lysosomal compartments (Figure 4B). Even if this inward flow of membrane caused by cytoplasmic dynein activation is gradually restored to the steady state, the initial imbalance could be sufficient to promote increased accumulation of drug in late endosomes or

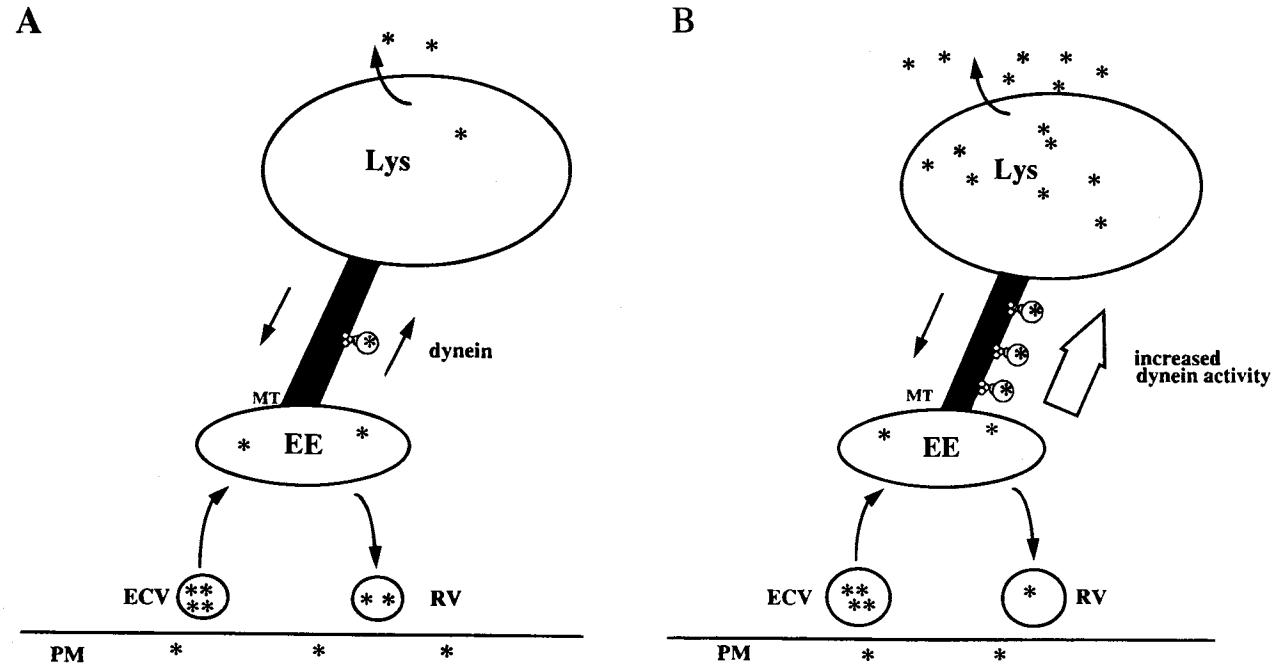

Fig. 4. Hypothetical scheme for the use of cytoplasmic dynein activation to enhance drug delivery to lysosomes. A. Under normal conditions, most of the drug internalized to the early endosomes is recycled directly to the plasma membrane with a small percentage of drug moving to late endosomes and lysosomes in a cytoplasmic dynein-driven transport process. B. By activating cytoplasmic dynein, increased membrane movement from early to late endosomes and lysosomes occurs, carrying more drug to later compartments. This drug diffuses out of the lysosomal compartments, causing the intracellular concentration of drug to be increased. * indicates drug. EE, early endosome; Lys, lysosomes; ECV, endocytic vesicle; RV, recycling vesicle; PM, plasma membrane.

lysosomes, ultimately resulting in increased cytosolic concentrations of drug by diffusion from late endocytic and lysosomal compartments. Since targeted delivery of oligonucleotides and genes complexed to asialoglycoproteins via asialoglycoprotein receptors has been achieved in hepatocytes (1–3, 7, 8), and a direct role for cytoplasmic dynein in sorting of asialoglycoproteins from asialoglycoprotein receptor has been demonstrated (27, 29), cytoplasmic dynein activation to promote increased transport to the late endosomal and lysosomal pathway would appear to be a legitimate strategy for enhancing drug delivery.

Although not discussed in this review, MT-dependent transport pathways are also targets for enhancing drug transport across barrier tissues such as intestinal and lung epithelia and the blood brain barrier. Endocytosis and recycling at basolateral and apical plasma membranes in polarized cells can proceed as shown in Figure 1; in fact, drug delivery using the asialoglycoprotein receptor to mediate endocytic uptake of genes and oligonucleotides coupled to asialoglycoproteins occurs at the basolateral surface of hepatocytes (1–3, 7, 8), which are polarized cells. However, the movement of membranes and their contents from the basolateral to apical surface and from the apical to basolateral surface during transcytosis also occurs in polarized cells. Since epithelial and endothelial barriers are major impediments for the delivery of polar drugs from the gastrointestinal tract to the blood and from the blood to the brain, respectively, attempts at enhancing the transcytosis of proteins and peptides in polarized cells have been explored in depth (reviewed in 47). Since several studies support a role for MTs and MT-dependent motor proteins in transcytosis (reviewed in 12), additional information about the role played by MT-dependent motors in transcytosis may lead to new strategies for using MT-dependent motors to enhance transcytosis of drugs that are analagous to the hypothetical design shown in Figure 4.

This article focuses primarily on regulation of the MT-dependent motor proteins responsible for driving vesicle movement along the MT as a means of moderating intracellular membrane traffic and increasing uptake of extracellular components including polar drugs. However, it is possible that alterations in traffic could also be elicited through changes in the interphase MT array. Most investigations in this area have focused on the use of MT-targeted drugs that alter the extent of MT polymer present in the cells. Although drugs which promote MT disassembly (nocodazole, colchicine) and MT assembly (taxol) have been shown to impact on membrane traffic, their use is not practical as a drug-delivery strategy. This is because many of these agents are potent chemotherapeutic agents, used to block cell division of tumor cells by interfering with dynamics of the MT mitotic spindle. Therefore, these MT-targeted drugs are systemically toxic to dividing cells. Even if the toxicity of these drugs could be moderated, other studies have shown that endocytic uptake of receptors is suppressed rather than increased following treatment with MT-targeted drugs (14, 48). Little is known about the identity and regulation of proteins which may bind to intact interphase MTs and alter their ability to sustain cytoplasmic dynein and kinesin-driven vesicle transport. Just as kinesin and cytoplasmic dynein now appear to be viable targets for enhancing drug delivery, it is possible that future studies may identify new MT-binding proteins that can moderate MT-based vesicle transport and constitute new future targets.

CONCLUSION

It is evident from the literature that improved delivery of drugs to specific targets can be achieved through use of the existing cellular transport machinery, most commonly, receptor-mediated endocytosis (1–8). Since MT-dependent vesicle transport is now known to play a role in membrane traffic and endocytosis, the proteins involved in MT-based vesicle transport, particularly cytoplasmic dynein and kinesin, constitute new targets for enhancing drug delivery. Future research in the area of MT-based vesicle transport that may have potential for direct application to drug delivery will be on the mechanism of regulation of MT-based motors, particularly cytoplasmic dynein, in target tissues of interest. These studies will necessarily correlate measurements of motor activation in vitro (obtained through in vitro motility assays using video microscopy) with parameters of endocytic uptake in intact cells (increases in fluid-phase or receptor-mediated endocytosis). Further investigations on intracellular sorting within the endocytic pathway, and of the impact of MT-based motor activation on the sorting process will also be essential.

It is important to note that MT-dependent motors are only one component of the membrane traffic machinery. Many other components of endocytosis and membrane traffic also constitute potential targets for enhancing drug delivery including rab proteins, ARF1, clathrin, and heterotrimeric G proteins. It may be true that upregulation of these additional components of membrane traffic occurs in concert with upregulation of cytoplasmic dynein or kinesin activity. However, it is clear that by exploring the molecular mechanisms by which cytoplasmic dynein and kinesin-driven vesicle transport are regulated, and how changes in MT-based motor activity impact on the other component proteins involved in membrane trafficking, we may be able to specifically increase the uptake, alter the sorting, or prolong the retention time of polar drugs in target cells.

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