Experimental models to evaluate the role of P-glycoprotein in the blood–brain tumor barrier

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Abstract. The blood–brain barrier (BBB) is formed by the capillary endothelium in the brain. Tight junctions, low pinocytic activity and lack of fenestrae are hallmarks of this barrier that force compounds to enter the brain by transcellular passage of the endothelial cells. However, many drugs that have appropriate lipophilicity for passive diffusion are a substrate of the multidrug transporter P-glycoprotein (P-gp), which acts as a gatekeeper preventing their entry into the brain. The role of the BBB and of P-gp in the BBB is well established and it is clear it efficiently protects the brain against potentially hazardous substrates. The importance of P-gp in protecting brain tumors, however, is not so well defined yet. In this paper we will discuss our ongoing efforts to resolve this issue. We are developing a panel of experimental brain tumors in mice featuring intact and/or minimally disrupted BBB properties for studying the efficacy of chemotherapy against brain tumors. By using stereotactic implantation of tumor cells into the brain lesions arise with infiltrative, invasive and/or expansive growth characteristics as seen in brain cancer. Tumor cells have been tagged with luciferase to allow convenient non-invasive follow up of tumor burden. To evaluate the role of P-gp we have established colonies of P-gp knockout nude mice and wild-type nude controls to be used as recipients for these xenograft models. We have determined the pharmacokinetic behavior of the model P-gp substrates and potent anticancer drugs paclitaxel and docetaxel, given alone or in combination with P-gp inhibitors. We expect that docetaxel will be most informative on the role of P-gp, because the absence of P-gp does not alter the plasma clearance of this drug. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

It is well established that the blood–brain barrier (BBB) that is formed by the capillary endothelial cells in the brain limits the brain entry of potentially harmful substances. The low permeability of the vessel wall mainly results from two important factors being: a) the presence of tight junctions restricting paracellular transport, and b) a low endocytic activity of the cerebral capillary endothelium. Essential nutrients such as glucose are delivered to the brain by selective transport mechanisms, but most other substances can only enter the brain via transcellular passive diffusion through the endothelial cells.

Many drugs that have appropriate lipophilicity for transcellular diffusion, however, are a substrate of the multidrug transporter P-glycoprotein (P-gp), which acts as a gatekeeper preventing those compounds from entering into the brain [1]. It is well established that P-gp in the BBB protects the brain against the entry of potentially toxic substrates. The importance of P-gp in the blood–brain tumor barrier (BBTB), however, is not very well defined. On the one hand it is known that the vasculature of brain tumors is locally disrupted and leaky, suggesting that the BBTB is not very stringent. On the other hand, however, primary brain cancer cells are highly infiltrative and may diffuse centimeters away from the central and leaky areas of the tumor into the normal surrounding brain parenchyma, where they may be protected by an uncompromised BBB. Similarly, many extracranial tumors such as breast cancer and melanoma metastasize to the brain at high frequency and metastatic seeds from such lesions may also find themselves shielded from chemotherapeutics by a functional BBB as long as they have not grown to lesions of a size requiring new blood vessel formation for oxygenation and nutrition.

Recently, Fellner et al. [2] by using an intracranial tumor model showed that the antitumor response of the P-gp substrate drug paclitaxel was improved when it was given in combination with the P-gp inhibitor valspodar (PSC833) and they came to the conclusion that P-gp in the BBTB discounts the efficacy of paclitaxel against intracranial tumors. However, several issues may limit the validity of this interpretation. Valspodar substantially reduces the plasma clearance of paclitaxel and the enhanced drug exposure of tumor tissue by itself may be an important reason for the higher efficacy, especially since the tumor vessels in their U118MG tumor model may exhibit compromised BBTB properties. Typically, malignant glioma derived tumor cells, such as the U118MG cell line, grow expansively as uniform lesions with sharp defined borders, rather than in the diffuse and infiltrating fashion as is characteristic for gliomas in patients. Due to their expansive growth, such tumors have to rely on the formation of new blood vessels rather than using the pre-existing brain vasculature and these new vessels are generally considered to be very leaky.

To unambiguously establish the role of P-gp in the BBTB we are developing intracranial tumor models that have intact or minimally disrupted BBTB properties. These models are established in nude mice that are either proficient (wild-type) or deficient in P-gp (knockout) and will be treated with docetaxel since the plasma clearance of this drug is similar in wild-type and P-gp knockout mice. To make these tumor models more amenable for medium-throughput drug intervention studies, we have transfected the cell lines with the firefly protein luciferase as this allows the non-invasive in vivo follow up of the tumors by using a super-cooled camera.
2. Choice of model substrate drugs

We have investigated the usefulness of taxanes for our studies since these agents are good substrates of P-glycoprotein and because these drugs have demonstrated good efficacy against many extracranial malignancies. By determining the disposition in wild-type and mdr1a-deficient FVB mice we have shown that P-gp is a major factor limiting the brain penetration of these drugs (Fig. 1). The brain exposure to paclitaxel was about 10-fold higher in P-gp knockout mice than in wild-type controls [3]. Moreover, the elimination of paclitaxel from the brain occurred very slowly as about half the level present at 24 h after drug administration was still present after 96 h. In our tests we also included a number of P-gp inhibitors that were given concomitantly with paclitaxel to wild-type mice in order to investigate whether and to what extent these agents contribute to increase the brain levels of paclitaxel. So far, we have not been able to identify an inhibitor that has sufficient potency to fully inhibit P-gp in the BBB. However, elacridar (GF120918) appeared to be the best drug for this purpose, especially when given in repeated dosings [3]. This compound is now being tested in an ongoing clinical trial. Importantly, elacridar has also little effect on the plasma clearance of paclitaxel, except for the about 50% reduction that is caused by inhibition of P-gp in the organs that are involved in its elimination [4]. Clearly, valspodar that is also a potent P-gp inhibitor decreased the clearance much more pronounced, which may have compromised the interpretation of the results of Fellner et al. [2], as outlined in the introduction.

We have further investigated the brain penetration of docetaxel in wild-type and P-gp knockout mice [5]. The brain penetration of docetaxel was also about 10-fold higher in P-gp knockout mice than in wild-type controls (Fig. 2). In a separate study we had already found that the plasma clearance of this compound is not affected by the absence of P-gp because the elimination of this drug proceeds mainly by metabolism through cytochrome P450 enzymes [6]. This lack of a pharmacokinetic interaction is a major advantage since the interpretation

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Fig. 1. Brain penetration and plasma levels of paclitaxel. Paclitaxel was given at a dose of 10 mg/kg to WT mice (○) and P-gp knockout mice (O), or given to WT mice in combination with elacridar (GF120918; ▲), valspodar (PSC833; ■) or cyclosporin A (■). Animals were killed at 1, 4, 8 and 24 h after drug administration and drug levels determined by HPLC. (See Ref. [3] for further details).
of efficacy data will not be obscured by differences in systemic drug exposure. Elacridar was also the best candidate P-gp inhibitor, as it did not alter the clearance of docetaxel, whereas valsapodar and cyclosporine had a clear effect on the plasma clearance.

2.1. Selection of tumor cell lines

Although our work is primarily focused on the treatment of malignant glioma it is for the purpose of this work not essential that the cell lines in our studies be of glial origin. Our main interest was to develop brain tumor models that leave the BBB virtually intact and this quality is usually not met when using glioma derived cell lines such as U87MG, U118MG and others. As an example of our work we here show the results of two cell lines that do show suitable growth characteristics (see Fig. 3). The first example is the K1735Br2-luc cell line that is derived from the murine melanoma cell line K1735-SW1, kindly provided by Dr. I.J. Fidler. It was previously demonstrated that the K1735-SW1 forms invasive lesions in the
brain parenchyma following intracarotid arterial injection [7]. The other example is the Mel57 human melanoma cell line, which was shown to be less invasive than the K1735, but leaves the BBB virtually intact when growing in the brain after intracarotid arterial injection [8]. We have now established that these invasive growth characteristics are maintained when these cells are stereotactically injected into the brain (Fig. 3).

2.2. Transfection with luciferase

Non-invasive monitoring of the intracranial tumors is a prerequisite for using them in therapy intervention studies. We have created brightly luminescent sublines that allow monitoring starting as early as 1–2 days after tumor cell injection. All cell lines have been transfected using a plasmid containing the luciferase gene coupled via an internal ribosome expression site to GFP and a neomycin selection cassette. GFP bright cells were sorted to single cells by FACS, expanded and tested for luciferase expression by an in vitro bioluminescence assay (Promega). Four to 5 sublines of each cell line showing the highest luminescence activity were tested in vivo for tumor take and intracranial growth behavior (Fig. 4).

2.3. Establishment of intracranial tumors

We have chosen to intracranially inject the tumor cells with a stereotactic device and an infusion pump rather than using intracarotid injections, because tumor growth in this way

Fig. 4. Bioluminescence imaging of intracranial tumors. Tumor burden is quantified based on the emitted photon flux after i.p. administration of 150 mg/kg of luciferin. Animals are anesthetized using isoflurane and placed in the thermostatically controlled compartment. The exposure time depends on the amount of emitted light, but is usually between 5 s and 1 min.
proceeds in a highly standardized fashion with reasonable reproducibility in bioluminescence between animals and a good correlation of bioluminescence with tumor burden. A particularly troublesome complication when using intracarotid injections was the fact that extracranial tumor growth (in the facial muscles) occurred frequently and the light emission from these more supervisual tumors exceeded the light coming from the deeper lying intracranial tumors.

2.4. Animals

Athymic FVB mice kindly provided by Dr. Carl T. Hansen from the National Institute of Health (Bethesda, MD, USA) were crossed with our Mdr1ab deficient (P-gp knockout) mice previously backcrossed to a 99% FVB background [9]. The resulting F1 was backcrossed to obtain athymic FVB mice with homozygous wild-type alleles for Mdr1a and Mdr1b and knockout nude mice with homozygous deleted alleles for Mdr1a and Mdr1b. Next, we used conventional breeding to further expand these two mouse lines to yield colonies of P-gp wild-type (FVBnude) and of P-gp deficient (MDRnude) mice. Pairwise analysis of the antitumor efficacy of docetaxel against brain tumors growing in FVBnude and MDRnude will be the key to elucidate the importance of P-gp expression in the BBTB.

3. Conclusion and future perspectives

Experiments testing the efficacy of docetaxel against intracranial tumors are now ongoing. These tests should unambiguously establish whether the BBTB in general and P-gp in the BBTB in particular protect intracranial tumors from systemic chemotherapy. Brain cancer (malignant glioma) is among the deadliest of human cancers. Despite advances in the treatment of many other human cancers, the progress that has been achieved in the treatment of high-grade glioma is very modest. Now, in the era of molecular biology it is hoped and/or expected that insight in the derailed molecular pathways may result in the identification of novel targets and novel therapeutics for brain tumor therapy. Understanding the contribution of P-gp in the BBTB on systemic chemotherapy will be of major importance as, obviously, even the smartest drugs will have to bypass this barrier in order to reach into the diseased tissue.

References

Discussion

Du Souich
Probably glut-1 expression is increased simply by hypoxia, that is, by HIF (hypoxia inducible factor). In our hands with a completely different model—in vivo hypoxia—used in other type of studies, hypoxia does also increase PGP in vivo. Then, it’s not unexpected that the glut-1 is increased. NOS is increased and many other transporters are probably increased, all those modulated by HIF.

Van Tellingen
Of course, you are right in that respect. The only thing is that we haven’t noticed that in any other models, like the mammary breast cancer tumor, in which if you look at it in detail there is certainly evidence of hypoxia as well.

Du Souich
I think it depends on which region of the tumor you look at. Because if it is very close to the newly formed vessels, probably in zones where the hypoxia is lesser, then you may not find changes in protein expression.

Van Tellingen
I must say that the glut-1 expression is quite uniform all over the tumor in U87. It’s not primarily at those sites that are necrotic or hypoxic sites. But of course, the HIF one is a route that we have been thinking about.

Kreuter
To what extent does angiogenesis play a role in brain tumors? And following this line, can one use anti-angiogenesis drugs like thalidomide?

Van Tellingen
Of course, angiogenesis is very important in malignant glioma, no doubt about it, but as I explained, there are escape mechanisms present in the brain that may render those therapies much less efficacious than you would have hoped for in the first place. The whole area of neo-angiogenesis has been slowed down a little bit after the early enthusiasm and now has come up with the recognition that we have at least one antibody that is efficacious. But, besides that the brain may certainly be a very difficult area to treat because of the possibility of those escape mechanisms.

McQuaid
Do you know anything about the integrity of the neo-vasculature within these tumors? For instance, do the vessels express tight junctions?
Van Tellingen
In the Mel-57 these are tightly bound, as shown by the MRI. This is a real barrier for leakiness.

Sugiyama
In the case of extra-brain tumors many people have been using the so-called MDR1 modulator to enhance chemotherapy. In such cases, the dose of anticancer drugs can be decreased. However, without having some selectivity of that MDR1 modulator between the extra-brain tumors and the normal blood–brain barrier we can never get good chemotherapy. That’s my opinion. What do you think about that?

Van Tellingen
I do not think that the use of P-glycoprotein blockers to enhance the effect of chemotherapy of extracranial tumors will give better therapies. Tumor cells are too clever to rely on only one mechanism of resistance.

Whittle
Thalidomide has been used in malignant brain tumors, but it’s not very effective at all. The response rate is about 15%, but it’s used in a rescue–salvage phase, it’s not been used early in therapy. What about the cell line sensitivity to doxetaxel?

Van Tellingen
They are all sensitive, in vitro and also in extracranial tumor models. They are all sensitive to treatment with paclitaxel and/or doxetaxel. The key issue is: Are we able to treat those tumors very well when they are not behind the blood–brain barrier? What we are going to do is to show that the Mel-57 tumors that are intractable with our compounds now can be treated if we take the Mel57, the VHF transfected one, because in that case we have the same cell line and we are sure that we have a leaky blood–brain barrier there and we should be able to treat those tumors as well.

Whittle
What does the sensitivity of the wild-cell type compare with cell lines transfected with luciferase?

Van Tellingen
There’s no difference. We usually get a lot of single cell clones and we test them in different screens to make sure that they are still the same as the original cell line from which they derived. For example, the reason the reason why we didn’t work so much with the K1735 Q735, the melanoma cell line, is because it expresses a low level of endogenous P-glycoprotein, so that makes this cell line less sensitive to paclitaxel and doxetaxel. Also, if you are going to work with P-glycoprotein blockers, you don’t know if you are blocking P-glycoprotein in the blood–brain barrier or blocking P-glycoprotein in the tumor cells themselves. That complicates matters. But I think Mel-57 is clearly PGP negative and it has all the characteristics you would like to have to show the importance of the blood–brain barrier.