Signaling in oligodendrocyte progenitor cells.
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Multiple sclerosis (MS) is the most well-known demyelinating disease in humans. Although its main cause is still not known, environmental (viral infections), autoimmunity and genetic factors have been implicated in the etiology of MS. Therefore, an effective therapy for MS is not available yet, but current treatments are directed toward preventing myelin breakdown via suppression of the immune system. However, promotion of myelin repair via remyelination-based strategies after or during treatment of the inflammatory response may become equally important. To promote remyelination in MS lesions via either endogenous or transplanted oligodendrocyte progenitors, it is necessary that oligodendrocyte progenitors migrate, proliferate and eventually differentiate to mature myelinating oligodendrocytes at the site of the MS lesion. Alternatively, remyelination by the remaining mature oligodendrocytes may be induced. To guide (re)myelination in MS lesions and to obtain an effective remyelination-based therapy, it is imperative to understand the key events in the process of myelination. Such events include the extracellular and subsequently activated intracellular events that are important during oligodendrocyte differentiation and the onset of myelination. The goal of the project, described in this thesis, was therefore to study the involvement of both extracellular and intracellular signals in oligodendrocyte lineage progression.

**Intracellular signaling: PKC signal transduction pathway**

In rats, oligodendrocyte progenitors migrate, proliferate and differentiate in vivo according to a precise schedule over a period of several weeks in order to myelinate axons at the appropriate time and place. This implies a tightly controlled mechanism in order to prevent premature and nonessential differentiation. The study described in chapter 2 showed that the protein kinase C (PKC) signaling route is an important negative modulator in the regulation of the switch to differentiation. We also demonstrated that activation of PKC prevents (premature) oligodendrocyte differentiation, whereas further differentiation toward myelinating oligodendrocytes is dependent on other signals, like elevation of cAMP levels, which may counteract the PKC signal transduction pathway. In chapter 3 it was revealed how activation of PKC keeps the oligodendrocyte progenitors arrested in the pro-oligodendrocyte stage. This is accomplished by regulation of the actin cytoskeleton structuring via phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS). Upon PKC-mediated phosphorylation, MARCKS was translocated from the plasma membrane to the cytosol, and consequently no longer capable of regulating actin-membrane interactions. As a result, actin filaments are redistributed to the submembranous or cortical actin cytoskeleton, which caused an impediment of plasma membrane-directed traffic of 'basolateral'-sorted proteins. On the other hand, under the same conditions, PKC activation stimulated the transport of 'apical'-predestined proteins to specific sites just underneath the plasma membrane. Another PKC target is probably involved in this differential control of cognate polarized trafficking, as it did not interfere with plasma membrane-directed traffic of oligodendrocyte progenitors.

**Extracellular signaling**

To establish an extracellular signal transduction pathway that was not inhibited by PKC activation, basic fibroblast growth factor was used as a mitogenic signal. By co-culture with PKC-selected oligodendrocyte progenitors, oligodendrocyte proliferation was stimulated. To establish the regulation of this mitogenic signal with PKC-signaling, we studied the effect of PKC on proliferative and differentiative events. As a result, we concluded that oligodendrocyte lineage progression is partly regulated by mitogenic signals. Such a signal is partly able to partly activate PKC.

**Signaling in differentiating oligodendrocytes**

To study the role of PKC activation during oligodendrocyte differentiation events, an in vitro model was used. Differentiated oligodendrocytes and neurons and astrocytes were cocultured as closely as possible. PKC or basic fibroblast growth factor were added. Expression of PLP was induced after basic fibroblast growth factor was expressed.
Although its genetic factors for MS is not breakdown via remyelination, become equally transplanted rate, proliferate become induced. To therapy, it is important during project, described and intracellular polarized trafficking pathways, as direct perturbation of the actin cytoskeleton perturbation did not interfere with the translocation of 'apical'-like proteins. Final incorporation into the plasma membrane might be hindered by a lack of appropriate docking devices in oligodendrocyte progenitors.

**Extracellular signaling: PDGF and bFGF signal transduction pathways**

To establish the physiological relevance of the PKC-related effects, we investigated whether or not inhibition of oligodendrocyte differentiation by platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) was related to PKC activation. In chapter 4, it is described that both PDGF and bFGF induce phosphorylation and translocation of the PKC substrate, MARCKS, and as a consequence induce redistribution of actin filaments to the cortical actin cytoskeleton. This indicates that PKC activity is instrumental in inhibiting oligodendrocyte differentiation by both growth factors. Yet, since the induction of proliferation was not PKC-mediated, differentiation was still inhibited after pre-treatment with PKC-specific inhibitors. Hence, to induce differentiation in PDGF- or bFGF-activated oligodendrocyte progenitors a simultaneous abrogation of the growth factor-induced proliferative activity is necessary. The mitogenic activity of PDGF and bFGF was blocked by pre-exposure to inhibitors of p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, and pp70 S6 kinase, while the activity of these kinases was essential for the onset of oligodendrocyte differentiation as well. We suggest therefore, that oligodendrocyte lineage progression is regulated by independent signaling pathways, acting in parallel and steering mitogenic activity and/or differentiation arrest. As a consequence, oligodendrocyte maturation, and subsequently myelination, is accomplished by the action of two or more signals acting in concert that counteract PDGF- and/or bFGF-activated signaling pathways.

**Signaling in an in vitro myelinating culture**

To study the significance of regulation of oligodendrocyte differentiation via modulation of signaling events with respect to (re)myelination in MS lesions, we introduced and optimized an *in vitro* myelinating system derived from fetal rat brain. In these cultures astrocytes, neurons and oligodendrocyte are present, and thereby mimicking the *in vivo* situation as closely as possible. Using this *in vitro* myelinating system, we describe in chapter 5 that when PKC or bFGF signaling routes were activated during development, oligodendrocyte differentiation was inhibited. The expression of the late myelin-specific proteins MBP and PLP was impeded, in contrast to the early myelin-specific proteins CNP and MAG that were expressed. Accordingly, myelin formation was absent in these cultures.
formation was also absent upon continuous occupancy of PDGF receptors during development, even though CNP, MAG, MBP and PLP were expressed in normal amounts in these cultures. This indicates that differentiation per se does not inherently lead to myelination. We anticipate that this in vitro myelinating system will provide further insight into the regulation of differentiation and the onset of myelination. Preliminary experiments have also shown that it is possible to design a demyelinating model of this in vitro myelinating system, thus allowing a detailed study of the signaling events required for remyelination.

Identification of functional caveolae in the oligodendrocyte lineage

A mechanism to control asynchronous oligodendrocyte differentiation may imply a localized signaling via caveolae, which are known as organized signaling centers at the plasma membrane of mammalian cells. In contrast to previous claims, the results described in chapter 6 demonstrate that caveolin-1, the major ‘caveolar coat’ protein, is expressed in a oligodendrocytic cell line (OLN-93) and even in primary oligodendrocytes, albeit to a lesser extent. Furthermore, we demonstrated that functional caveolae were present in the oligodendrocyte lineage. However, electron microscopic analysis and cholera toxin subunit B (CTB) internalization assays showed that not every cell in the oligodendrocyte lineage assembled caveolae. Indeed, indirect immunofluorescence microscopy revealed that the majority of caveolin-1 was intracellularly located in oligodendrocytes. In this regard, the data indicate that functional assembly of caveolae is cell-cell contact-dependent. Therefore, it is possible that the critically timed regulation of oligodendrocyte differentiation is accomplished via local and organized signaling in caveolae. Furthermore, since caveolin-1 and caveolae has been implicated in polarized traffic events and given its intracellular localization, a role for caveolin-1 in vesicular trafficking in the oligodendrocyte lineage is suggested.

General conclusions

Taken together, the studies described in this thesis have provided insight into the complex regulation of oligodendrocyte lineage progression. The results obtained can be summarized in two fairly simplified models, describing (i) intracellular signaling events involved in oligodendrocyte differentiation (fig. 1A) and (ii) the complex interplay between extracellular and intracellular signaling events, leading to proliferation and/or differentiation (fig. 1B). Briefly, oligodendrocyte differentiation can be blocked by PKC activation or by p42/p44 MAPK, p38 MAPK or pp70 S6 kinase inhibition (fig. 1A). There seems to be no cross-talk between signaling pathways activated by the particular PKC signaling route and these other kinases. PKA activation can negatively interfere with PKC-mediated inhibition of oligodendrocyte differentiation. The PDGF- and bFGF-mediated inhibition of oligodendrocyte
Summary and perspectives

Figure 1: Schematic model of signaling factors involved in oligodendrocyte differentiation and/or proliferation. Both models are based on results obtained in studies, described in this thesis. A) intracellular signaling events in oligodendrocyte differentiation; B) extracellular activation of intracellular signaling events in both oligodendrocyte differentiation and proliferation.
Chapter 7

differentiation occurs at least in part via PKC activation (fig. 1B). Yet, differentiation has to be accomplished by PKC inhibition, and a simultaneous abrogation of growth factor-induced proliferation via inhibition of either p42/p44 MAPK, p38 MAPK, or pp70 S6 kinase. However, as these latter signaling molecules are also involved in the induction of oligodendrocyte differentiation (fig. 1A), the length of the period of activation of these kinases is probably important for determining whether proliferation or differentiation will take place. Transient versus sustained activation can be accomplished via cross-talk with the PKA signaling pathway, as PKA activation can induce oligodendrocyte differentiation to the next developmental stage in PDGF-treated progenitors, and significantly inhibits bFGF-mediated proliferation.

Insight has also been obtained with respect to possible approaches in the development of remyelination-based strategies. Although studies were performed with rat-derived cultures, the results obtained will certainly facilitate research in human. The absence of a significant degree of spontaneous remyelination in MS lesions may be due to either the presence of inhibitory factors preventing myelin repair or the absence of cells or factors necessary for de novo myelin synthesis. Possibly, a combination of signaling factors, likely including PKC-inhibitors, might be beneficial to the treatment of MS in the (near-)future. Still, extensive work is necessary to further elucidate the most promising set of signaling factors, capable of inducing (re)myelination in MS lesions. Not only the signaling factors involved in proliferation and differentiation of oligodendrocyte progenitors, but also those involved in migration and survival of oligodendrocytes are of crucial importance in this respect.

Perspectives

Future fundamental experimental research should be focused on the interaction between different signaling pathways, which provides specific and precisely timed regulation of oligodendrocyte differentiation. The essential asynchronous differentiation of oligodendrocytes is probably accomplished via integration of general and local signals, such as those exerted by astrocytes or neurons. Localized signaling, as a modulator of oligodendrocyte lineage progression, provides a means by which the intrinsic differentiation pathway can be modulated according to the need for oligodendrocytes at any particular location. Specifically, the cross-talk between growth factor- and integrin-mediated signaling in oligodendrocytes needs to be further explored, since the same signaling molecules as those in growth factor signal transduction pathways were activated upon extracellular matrix (ECM) binding and integrin clustering (Juliano, 1996). Furthermore, an indication that integrin-mediated signaling may play a significant role in modulating oligodendrocyte differentiation, is dictated by the switching of integrin β subunits during differentiation (Milner and ffrench-Constant; 1994). Moreover, there is some evidence that integrin- or ECM-mediated adhesion of oligodendrocyte progenitors may modulate, affect, or even arrest. Alternatively, it may modulate adhesion of hydrophobic gradients necessary for differentiation. The arrest induced by specific intracellular signaling pathways of these molecules may be necessary to examine the potential of treating MS with combinations of drugs.

Another intriguing point of significance in this regard provided the identification of signaling factors that have shown to differentiate or stimulate oligodendrocytes in vitro. For instance, the identification of factors that contribute to the onset of myelination may have implications for remyelination in MS lesions.
Summary and perspectives

of oligodendrocytes may trigger oligodendrocyte differentiation (Malek-Hedayat and Rome, 1994, Pesheva et al., 1997). Hence, triggering of integrin-induced signaling cascades may affect, or even counteract the effect of PDGF and bFGF on oligodendrocyte differentiation arrest. Alternatively, an interplay between growth factor- and hormone-mediated signaling may modulate the switch to oligodendrocyte differentiation. Barres et al. (1994b) reported that hydrophobic signals, like thyroid hormone (T3), glucocorticoids and retinoic acid, are responsible for timing oligodendrocyte differentiation by counteracting the differentiation arrest induced by mitogenic growth factors. As these hydrophobic signals bind directly to intracellular receptors that activate transcription, a cross-talk between these signaling pathways on a transcriptional level is likely. Interestingly, these hydrophobic signaling molecules share the ability with PKC, PDGF and bFGF to modulate the activity of the transcription factor, activator protein-1 (AP-1). It will be of particular interest, therefore, to examine the effects of PKC-, bFGF- or PDGF-activated signaling routes, alone or in combination with hydrophobic signals, on transcriptional activity.

Another intriguing question for future research is whether PKA activation is of physiological significance in the timing of oligodendrocyte differentiation. In this thesis, evidence has been provided that PKA activation can partially counteract PKC-mediated arrest of oligodendrocyte differentiation and bFGF-induced proliferation. Furthermore, Raible and McMorris (1993) have shown that PKA independently upregulates the rate of differentiation of oligodendrocyte progenitors, and inhibits their proliferation without changing lineage commitment. Therefore, the identification of extracellular upstream signaling events in relation to PKA activation will contribute to the unraveling of signaling events involved in the timing of oligodendrocyte differentiation. In this respect, and also in the context of facilitation of (re)myelination, more attention should be paid to signaling events exerted by other positive modulators of oligodendrocyte differentiation, including insulin-like growth factor (IGF, McMorris and Dubois-Dalcq, 1988) and ceramide (our unpublished observations).

To elucidate the exact sequence of molecular events in oligodendrocyte differentiation and the onset of myelination, the availability of appropriate model systems will be imperative. The in vitro myelinating system described in this thesis (chapter 5) may be a valuable tool in the characterization of these events. Ultimately, this knowledge of signaling events, important for the onset of myelination, may lead to a promising strategy to promote myelin repair in MS lesions.