Article available at http://www.parasite-journal.org or http://dx.doi.org/10.1051/parasite/2008153286

THE INFLUENCE OF HOST HORMONES AND CYTOKINES ON *ECHINOCOCCUS MULTILOCULARIS* SIGNALLING AND DEVELOPMENT

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Summary:

Parasitic helminths display highly complex life-cycles in which the establishment of adults or larvae within host target organs as well as the transition of one developmental stage to the following is influenced by host-derived factors. Due to its approachability concerning *in vitro* cultivation, the larval stage of the fox-tapeworm *Echinococcus multilocularis* has recently emerged as a model system to study the molecular nature of such host-derived stimuli and their influence on developmental control in the parasite. Data obtained so far indicate that cytokines which are used by the host for cell-cell communication can also be exploited by the parasite as clues to find suitable target organs. This involves direct interactions of evolutionary conserved signalling systems of the receptor tyrosine – and the receptor serine/threonine-kinase pathways of the parasite with corresponding host cytokines of the insulin –, the epidermal growth factor-, and the transforming growth factor-β-families. In the present article, we will briefly review *in vitro* cultivation approaches undertaken so far for *E. multilocularis* larvae as well as our current knowledge on the parasite's signalling systems and their interaction with host cytokines.

KEY WORDS : *Echinococcus*, cestode, parasite, signalling, *in vitro* cultivation.

Among the diverse groups of infectious agents (*e.g.* bacteria, fungi, viruses, protozoa), parasitic helminths are in so far unique as they rely on developmental mechanisms which have arisen early in metazoan evolution and are, therefore, also present in their various metazoan hosts. One of the earliest steps in animal evolution was the development of cell-cell communication mechanisms and the basic toolkit of respective systems was already established in the most primitive, sponge-like animals. Hence, hormones and cytokines of the insulin-, the epidermal growth factor (EGF)-, the fibroblast growth factor (FGF)-, or the transforming growth factor-β/bone morphogenetic protein (TGF-β/BMP)-families which signal through receptor tyrosine- and receptor serine/threonine kinases (RTKs, RSKs) are used for cell-cell communication in a wide variety of pre-metazoan and metazoan animals as different as cnidaria, insects, nematodes and mammals (Pires-da Silva & Sommer, 2002). Not only show insulin-, EGF-, FGF- and TGF-β/BMP-like cytokines from different animal phyla clear structural homologies, they can also functionally replace each other. This has been shown through early studies on classical animal models such as *Drosophila melanogaster* and *Caenorhabditis elegans* in which mammalian insulin- and BMP-cytokines were able to stimulate respective surface kinases of the insulin- and the TGF-β/BMP-receptor families of the invertebrate species (Kingsley, 1994; Fernandez *et al.*, 1995). Although it does not occur very often in nature that invertebrates come into contact with human insulin, the situation drastically changes when systemic helminths (filaria, schistosomes, cestode larva), which develop in close contact with the host's endocrine and paracrine system, are considered. In this setting, the structural and functional conservation of animal cell-cell communication systems raises several important questions: 1) Do systemic helminths also express evolutionary conserved signalling systems (accessible to host cytokines)? 2) If so, can these parasite signalling systems functionally interact with surrounding host hormones/cytokines and does this have an influence on parasite development? 3) Could parasitic helminths use specific cytokine signatures of organs or tissues as a means to 'find their way' within the host? 4) If hormonal cross-communication between parasite and host occurs, is it a common principle for all helminths or do only a few employ this strategy?

As briefly outlined below, several research groups currently address these questions using trematodes such as *Schistosoma mansoni* and nematodes such as *Brugia malayi* as model systems. We have, for several good reasons, chosen the cestode *E. multilocularis*.

THE *E. MULTILOCULARIS* LIFE-CYCLE *IN VIVO* AND *IN VITRO*

The adult stage of the tapeworm *E. multilocularis* resides within the intestine of the definitive host (*e.g.* foxes or dogs) and produces infective eggs which contain the oncosphere larva. The eggs are relea-

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sed into the environment through fox-faeces and are orally taken up by intermediate hosts such as small ruminants (or, accidentally, humans), whereupon the oncosphere hatches and penetrates the intestinal barrier. Almost exclusively within the liver of the intermediate host, the oncosphere undergoes a metamorphosis towards the metacestode stage which is a bladder like structure that infiltratively grows into the surrounding liver tissue. At a certain time point of the infection, multiple protoscoleces, which are the head regions of the future adult tapeworms, develop within the metacestode and are passed on to the definitive host when it takes the intermediate host as a result of natural predator-prey relationships (Brehm *et al.*, 2006). The big advantage of *E. multilocularis* over related, human-parasitic species such as *E. granulosus* or *Taenia solium* is that it can be relatively easily kept in the laboratory through serial intra-peritoneal passages in rodents (Siles-Lukas & Hemphill, 2002). Furthermore, and very important for investigations on host-parasite interaction, attempts to in vitro cultivate *E. multilocularis* larvae were successfully carried out in the past. Currently available are methods for the *in vitro* activation of the oncosphere and its metamorphosis towards the metacestode (Deplazes & Gottstein, 1991) as well as systems in which metacestode vesicles, cultivated in the presence of host feeder cells, continually grow and eventually produce protoscoleces (Hemphill & Gottstein, 1995; Jura *et al.*, 1996). In combination, these systems allow a complete in vitro-reconstruction of the developmental steps that occur within the intermediate host. Moreover, all studies on these systems indicated that factors which are secreted by cells of the intermediate host are governing parasite development. However, for investigations concerning the influence of defined host factors on the parasite, these systems are of limited use due to the continuous presence of host cells. This led us to develop an axenic *in vitro* cultivation system in which metacestode vesicles can be kept for prolonged periods of time in the complete absence of host cells (Spiliotis *et al.*, 2004). In this system, which involves cultivation of the parasite larvae under reducing conditions (β-mercapto-ethanol; nitrogen gas phase), parasite growth and protoscolex development can be obtained when conditioned medium from host cells is used, whereas no growth occurs in the presence of host serum. Using the axenic cultivation system it is now possible to add a variety of defined host factors to serum-containing medium and to test their specific effects on parasite development. Although our respective investigations are still ongoing, data gathered so far clearly indicate that host cytokines which stimulate RTKs, such as insulin, EGF or FGF, all have positive effects on parasite growth whereas cytokines of the TGF-β/BMP-family appear to inhibit metacestode growth and rather stimulate protoscolex development (our own unpublished results). Hence, cytokines and hormones which are involved in cell-cell communication in mammals do have clear effects on the development of the parasitic invertebrate *in vitro*. It is interesting to note that all the above mentioned cytokines and hormones which stimulate parasite development *in vitro* are also present in considerable amounts in the mammalian liver. Therefore, the specific outgrowth of the *Echinococcus* metacestode in the host liver could be initiated by the action of liver-typical, mammalian cytokines. To isolate parasite receptors for these cytokines, we subsequently started with genetic analyses as described below.

E. MULTILOCULARIS SIGNALLING SYSTEMS AND THEIR INTERACTION WITH HOST FACTORS

Using degenerative PCR approaches, we could identify a variety of RTK- and RSK-signalling factors of *E. multilocularis* and, at least partially, characterized these on the biochemical level (Table I). The parasite's signalling repertoire includes RTKs such as EmIR, an insulin-receptor like tyrosine kinase, and EmER, a tyrosine kinase of the EGF receptor family, which both display significant structural homologies to the respective receptors from mammals. Both RTKs are expressed in *Echinococcus* larval stages during the infection of the intermediate host in a manner accessible to host cytokines (Konrad *et al.*, 2003; Spiliotis *et al.*, 2003). A third *Echinococcus* RTK, EmFR, which is a member of the FGF-receptor family, has also recently been identified by us (Schäfer *et al.*, unpublished results) but not yet investigated in biochemical detail. One of the most important checkpoints for RTK signalling in animal cells is the mitogen activated protein (MAP) kinase cascade which, in mammals, involves small GTPases of the Ras-family as well as intracellular serine/threonine kinases such as Raf, MEK and Erk (see references in Spiliotis *et al.*, 2005, 2006). As could be expected from the presence of RTKs in *E. multilocularis*, the parasite larvae also express several MAP kinase cascade factors, particularly the small GTPase EmRas as well as the intracellular serine/threonine kinases EmRaf and EmMPK1, which all display considerable structural and functional homologies to MAP kinase cascade molecules from mammals (Spiliotis *et al.*, 2005, 2006). It is interesting to note that the *E. multilocularis* MAP kinase cascade factors, despite their clear homologies to the mammalian counterparts, also display several structural differences which could be exploited to develop a new generation of anti-parasitic drugs (Spiliotis *et al.*, 2005, 2006). A wide variety of substances which inhibit intracellular serine/threonine kinases is available

Protein	Gene	Function	Size*	% homol§	Reference
EmIR	*emir*	RTK; insulin receptor family	190	30(47)	Konrad *et al.*, 2003
EmER	*emer*	RTK; EGF receptor family	175	30(40)	Spiliotis *et al.*, 2003
EmRas	*emras*	Small GTPase; Ras family	21	79	Spiliotis *et al.*, 2005
EmRal	*emral*	Small GTPase; Ras family	23	53	Spiliotis & Brehm, 2005
Em1222	*emrab1*	Small GTPase; Ras-related	23	59	Brehm *et al.*, 2000
EmRaf	*emraf*	MAP-KKK; Raf family	87	41	Spiliotis *et al.*, 2005
EmMPK1	*emmpk1*	Erk-like MAP kinase	42	70	Spiliotis *et al.*, 2006
PDZ-1	*empdz1*	PDZ-domain scaffolding factor	23	51	Hubert *et al.*, 2004
Elp	*elp*	Ezrin-radixin-moesin like	61	46	Brehm *et al.*, 1999
Egfd	*emegfd*	EGF-like cytokine	8	33	Brehm *et al.*, 2003
EmTR1	*emtr1*	RSK; TGF-b type I receptor	61	34	Zavala-Gongora *et al.*, 2006
EmSmadA	*emsmadA*	Activin signalling; AR-Smad	35	60	Zavala-Gongora *et al.*, 2003
EmSmadB	*emsmadB*	BMP signalling; BR-Smad	48	62	Zavala-Gongora *et al.*, 2003
EmSmadC	*emsmadC*	Activin signalling; BR-Smad	37	81	Zavala-Gongora *et al.*, 2007
EmSmadD	*emsmadD*	TGF-β/BMP signalling; Co-Smad	77	45	Zavala-Gongora *et al.*, 2008
EmSkip	*emskip*	SNW/SKIP fam. transcr. coregul.	60	51	Gelmedin *et al.*, 2005

* protein size in kDa; § percent overall identity to corresponding human protein; value in brackets indicates identity in the tyrosine kinase domain.

Table I. – *E. multilocularis* signalling factors.

due to cancer research and could, after chemical modification, yield drugs which inhibit the parasite factor with higher specificity than the mammalian kinases. Preliminary data of our group already showed that several inhibitors designed against the mammalian p38 MAP kinase display higher specificity against the parasite orthologue EmMPK2 (Gelmedin *et al.*, manuscript in preparation).

Apart from RTK signalling components, evidence has also been obtained for the presence of RSKs of the TGF-β/BMP-receptor family in *E. multilocularis*. Molecular genetic and biochemical data for one of these receptors, EmTR1, have been published recently (Zavala-Gongora *et al.*, 2006). Similar to the situation for the *Echinococcus* RTKs, EmTR1 displayed significant structural and functional homologies to mammalian TGF-β/BMP-receptors, particularly to the ALK1 subfamily of type I receptors which are involved in BMP signalling. Molecular genetic evidence for three further TGF-β/BMP receptors, of which two belong to the type I subfamily and one to the type II subfamily, is also known, although the cytokine specificities of these RSKs have not yet been established in detail (our own unpublished results). Important downstream components of TGF-β/BMP signalling are the Smad transcription factors and at least four members of this family are expressed by *E. multilocularis* (Table I). One of these, EmSmadB, which belongs to the BR-Smad subfamily of receptor regulated Smads, functionally interacts with EmTR1 (Zavala-Gongora *et al.*, 2006), indicating that both proteins belong to an *Echinococcus* BMP signalling pathway. That the parasite also possesses a TGF-β signalling pathway is indicated by the structure of EmSmadA and EmSmadC which belong to the AR-Smad subfamily that usually mediates TGF-β and activin signalling (Zavala-Gongora *et al.*, 2003, 2008). Interestingly, the two *Echinococcus* AR-Smads differ from all other members of the protein family in that they lack a conserved MH1 domain. Furthermore, EmSmadA, despite being structurally an AR-Smad, obviously interacts with receptors from both the TGF-β and the BMP pathways (Zavala-Gongora *et al.*, 2003), yet again indicating slight differences in the intracellular RSK/RTK signal processing between *Echinococcus* and mammals. The final component, EmSmadD, is structurally a common mediator Smad which interacts with all three *Echinococcus* AR-Smads to form transcription complexes for TGF-β/BMP regulated genes (Zavala-Gongora *et al.*, 2007). Since EmSmadD is phosphorylated by the Erk-like MAP kinase EmMPK1, it apparently not only constitutes a central component of TGF-β/BMP signalling in *Echinococcus*, but also an important checkpoint for cross-regulation of RTK and RSK signalling in the parasite (Zavala-Gongora *et al.*, 2008).

Not only are the RTK and RSK signalling systems of *E. multilocularis* structurally homologous to the respective systems of the host, they are also capable of interacting with the corresponding host cytokines/hormones. In yeast two-hybrid experiments it has been demonstrated that EmIR interacts with human insulin in a manner comparable to that of the human insulin receptor (Konrad *et al.*, 2003). Human EGF, when added exogenously to *in vitro* cultivated metacestode vesicles, significantly stimulated the parasite's MAP kinase cascade and it is to be expected that EmER serves as the cognate parasite receptor for host EGF (Spiliotis *et al.*, 2006). Finally, upon heterologous expression of EmTR1 in human cells together with a BMP type II receptor, EmSmadB phosphorylation through EmTR1 could only be achieved in the presence of human BMP2, indicating that the parasite receptor directly interacts with the host cytokine (Zavala-Gongora *et al.*, 2006). Hence, the

suggested 'cross-communication' between evolutionary conserved signalling receptors of the parasite and corresponding host cytokines seems to occur in both RTK and RSK signalling.

THE SITUATION IN OTHER HELMINTHS?

Apart from *E. multilocularis*, considerable data on hormonal host-parasite cross-communication has been gathered for *S. mansoni*. This trematode expresses several RTKs of the insulin- and the EGF-receptor families (Dissous *et al.*, 2006) as well as surface RSKs of the TGF-β/BMP family (LoVerde *et al.*, 2007) which are considerably homologous to those of the host. Two of these systems, the EGF-receptor like RTK SER and the TGF-β receptor signalling complex formed by the type I/type II receptor pair SmTβRI/SmTβRII, are expressed on the surface of larval or adult schistosomes and interact with corresponding host cytokines, EGF and TGF-β, leading to specific responses in the parasite (Beall & Pearce, 2001; Vicogne *et al.*, 2004; Osman *et al.*, 2006). It is, therefore, to be expected that hormonal cross-communication between evolutionary conserved signalling systems of helminth parasites and their hosts is relatively widespread and forms a common principle of host-helminth interaction, at least in flatworms. An interesting variation on the theme has been observed in the parasitic nematode *Brugia malayi* which secretes a TGF-β cytokine orthologue, Tgh-2, into the surrounding medium (Gomez-Escobar *et al.*, 2000). Since Tgh-2 is able to activate the mammalian TGF-β receptor complex, its secretion by the parasite could be involved in modulating the host immune response through the TGF-β pathway.

CONCLUSIONS AND OUTLOOK

From the data obtained so far using *E. multilocularis* and *S. mansoni* as model systems, it appears clear that the concept of hormonal cross-communication between evolutionary conserved signalling systems plays an important role in host-parasite interaction in systemic flatworm infections. The currently ongoing genome sequencing projects for parasitic flatworms (Haas *et al.*, 2007; Aguilar-Diaz *et al.*, 2006) will surely yield further receptors and cytokines which add to the data already known for insulin-, EGF- and TGF-β/BMP receptors. Furthermore, the concept which so far mostly concentrated on peptide hormones and cytokines, should also be applicable to lipophilic hormones (*e.g.* steroids, vitamin A, vitamin D) which signal through the evolutionary conserved NHR family. Respective receptors with homologies to host counterparts have already been identified in *S. mansoni* (Wu *et al.*, 2005) and NHR signalling seems also to be present in *E. multilocularis* (Gelmedin *et al.*, 2005). That several of these flatworm NHRs functionally interact with lipophilic hormones of the host is to be expected and can be tested on the cellular level using the established in vitro cultivation systems. Unfortunately, it will be more difficult to obtain clear-cut *in vivo* evidence that hormonal cross-communication is important for helminth infections since transgenic mice which are fully defective in evolutionary conserved cytokines are mostly not viable. It will, therefore, be very important to develop methods for the genetic manipulation of parasitic helminths, which allow mutation-based approaches to study the role of signalling systems in the host-parasite relationship. For *S. mansoni*, respective methods are meanwhile available and are steadily improved (Kalinna & Brindley, 2007). For *E. multilocularis*, we have recently made the first step through the establishment of a primary cell cultivation system (Spiliotis *et al.*, 2008). In this system, totipotent germinal cells of the parasite are capable of fully regenerating infective metacestode vesicles within 5-6 weeks of cultivation. The transient introduction of foreign DNA into germinal cells as well as the stable transfection of germinal cells using virus-based systems has already been successfully carried out by us (unpublished data). Using the *in vitro* regeneration system, we are currently trying to produce fully transgenic parasite vesicles from these stably transfected primary cells. Once transgenic techniques are fully established, the role of RTK and RSK signalling systems in parasite development can be further approached using, for example, RNA interference or the expression of dominant negative receptor constructs.

ACKNOWLEDGEMENTS

We wish to thank the Deutsche Forschungsgemeinschaft (DFG) for supporting our work through grants SFB 479, BR2045/1-1, and the International Graduate College 1141 (all to K.B.).

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