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Inhibition of SERPINe1 reduces rhabdoviral infections in zebrafish

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ABSTRACT

While exploring the molecular mechanisms behind the fin hemorrhages that follow zebrafish (*Danio rerio*) early infection with viral haemorrhagic septicemia virus (VHSV), we discovered that most *serpin* (serine protease inhibitor) gene transcripts were upregulated, except those of *serpine1*. Surprisingly, only SERPINe1-derived 14-mer peptide and low molecular weight drugs targeting SERPINe1 (i.e. tannic acid, EGCG, tiplaxtinin) inhibited *in vitro* infections not only of VHSV, but also of other fish rhabdoviruses such as infectious hematopoietic necrosis virus (IHNV) and spring viremia carp virus (SVCV). While the mechanisms that inhibited rhabdoviral infections remain speculative, these and other results suggested that SERPINEe1-derived peptide specifically targeted viral infectivity rather than virions. Practical applications might be developed from these studies since preliminary evidences showed that tannic acid could be used to reduce VHSV-caused mortalities. These studies are an example of how the identification of host genes targeted by viral infections using microarrays might facilitate the identification of novel prevention drugs in aquaculture and illuminate viral infection mechanisms.

1. Introduction

In many fish, external (mouth, fin, epithelia) and internal (muscle, liver, head kidney, spleen, intestines) hemorrhages follow 4–7 days the start of rhabdoviral infections [1,2]. Although such rhabdoviral-induced hemorrhages should include interferences with the host molecular coagulation pathways, those have not been explored yet. Therefore, we were interested in the study of transcriptional expression profiles along the coagulation-complement pathway induced by rhabdoviral early infections in the fish fins, their portal of entry [3], with the expectation of finding novel gene targets for preventive drugs. We chose the zebrafish *Danio rerio* because of its susceptibility to novirhabdoviruses like viral haemorrhagic septicemia virus (VHSV) [4,5] or infectious hematopoietic necrosis virus (IHNV), coding for non-virion NV genes and to rhabdoviruses like spring viremia of carp virus (SVCV) which lack the NV gene [6,7]. In addition, zebrafish transcriptomic data on fins and lymphoid organs have been previously reported by our research groups after immunization with VHSV [8,9] and SVCV [10].

Vertebrate coagulation starts when damaged blood vessels expose collagens of internal tissues to blood proteolytic cascade factors (i.e. those coded by the *f3*, *f7*, *f8*, *f9*, *vwf* genes), leading to thrombin (*f2*) activation. Activated THROMBIN cleaves blood FIBRINOGEN (*fg*) generating the fibrin clots characteristic of coagulation (Fig. 1). The coagulation cascades are interconnected with parallel proteolytic cascades involved in complement-dependent cell lysis. Thus, the “complement and coagulation cascades” KEGG pathway (http://www.genome.ad.jp/kegg/) describes two proteolytic cascades interconnected by two proteins: FACTOR XII (endothelial secreted Hageman factor, coded by the *f12* gene) and PLASMINOGEN (a pro-enzyme, coded by *plg*). Proteolysis of FACTOR XII/PLASMINOGEN increase PLASMIN to reduce coagulation by fibrinolysis and to increase complement-dependent cell lysis (Fig. 1). To avoid tissue damage, excessive proteolysis are controlled by a variety of protein inhibitors (i.e.: coded by *serpind1*, *serpinc1*, *serpina1*, *serpinf2*, *serping1*, *serpine1*, *proc*, *tfpia*, *h*, *clu*, *etc*). Among them, serine protease inhibitors (SERPIN proteins) could be grouped in those inhibiting, i) Coagulation by targeting THROMBIN coded by the *f2* gene (SERPINd1, SERPINc1, SERPINa1); ii) Both coagulation and complement-dependent cell lysis by targeting TISSUE PLASMINOGEN ACTIVATOR, coded by *tpa* or *plaub* (SERPINa1, SERPINe1) and/or PLASMIN (SERPINa1, SERPINf2); and iii) Complement-dependent cell lysis by targeting C1S/C1R, coded by *c1s/c1r* (SERPINg1) (Fig. 1, red).

In this context, detailed re-analysis of previously published microarray gene results of the “complement and coagulation

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cascades" KEGG pathway of zebrafish fins infected with VHSV [8], discovered that most *serpins* were upregulated, except *serpine1*. Because the lack of modulation (up or downregulation) of *serpine1* could be explained by SERPINe1 interferences with VHSV infection, we then studied the effects of SERPIN specific inhibition on *in vitro* rhabdoviral infections. As demonstrated for human SERPINs [11,12], to specifically inhibit zebrafish SERPIN activities, we used the orthologous 14-mer amino-terminal amino acids of its Reactive Center Loops (RCL) ($_{NH2}$P14.P13.P12…P3.P2.P1…P'1.P'2.P'3…P'12.P'13.P'14). Surprisingly, we discovered that only zebrafish SERPINe1-derived peptides and/or their corresponding specific low molecular weight inhibitors (i.e.: tannic acid, EGCG, tiplaxtinin) [13,14] inhibited not only VHSV but also SVCV and IHNV *in vitro* rhabdoviral infections and reduced zebrafish mortalities of VHSV infections. This is one novel example of how the identification of host genes targeted by viral infections using microarrays might facilitate the identification of novel prevention drugs in aquaculture and illuminate viral infection mechanisms.

2. Materials and methods

2.1. Fish rhabdoviruses and cell culture

The viral haemorrhagic septicemia virus VHSV- 07.71 [15] and/or the infectious hematopoietic necrosis virus IHNV-Cedar strain [16] novirhabdoviruses were grown at 14 °C in the *Epithelioma papulosum cyprini* (EPC) cells from the fathead minnow *Pimephales promelas* (ATCC, CRL-2872). Cells were grown in 25 cm^2 flasks at 28 °C in RPMI-Dutch modified cell culture medium buffered with 20 mM HEPES (Gibco, Invitrogen corporation, UK) and supplemented with 10% fetal calf serum (FCS), 1 mM pyruvate, 2 mM glutamine, 50 μg/ml gentamicin and 2.5 μg/ml fungizone. To prepare VHSV or IHNV, the cell culture media were the same as above, except for the use of 2% FCS, 10 mM Tris pH 8.0 and 14 °C. To assay for inhibition of infectivity, VHSV or IHNV were purified by pelleting infected supernatants by ultracentrifugation at 60,000 *g* for 60 min at 4 °C and then titrated by a focus forming unit (ffu) assay

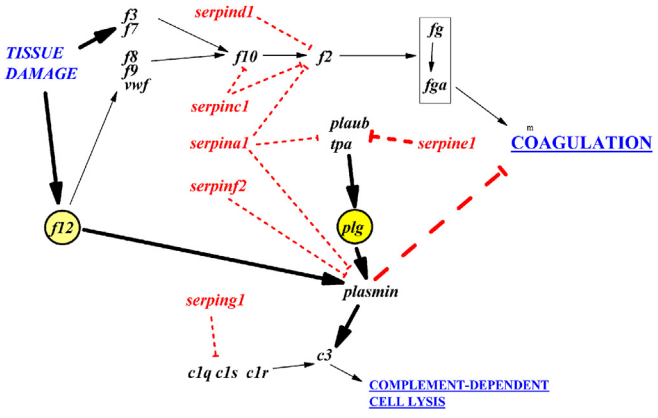

Fig. 1. Simplified scheme of the "Complement and coagulation cascades" KEGG pathway. The zebrafish gene symbols in *italics* and their proposed relationships were obtained from the corresponding human KEGG-pathway (http://www.genome.ad.jp/kegg/). ***Red italics***, Serine protease inhibitor (*serpin*) genes. All the human/zebrafish orthologous genes of the "Complement and coagulation cascades" pathway (Table 1) were assayed for transcript expression but only some of them have been drawn here for clarity. **Blue CAPITAL LETTERS**, main physiological inputs (cellular damage) and outputs (underlined, Coagulation and Complement-dependent cell lysis) of the pathway. Continuous black arrows, activation between gene products. **Discontinuous red arrows**, inhibition between gene products. Rectangle, fibrinogen conversion to fibrin. **Yellow circles**, *f12* and *plg* genes linking coagulation to complement proteolytic cascades.

using monoclonal antibodies to recognize VHSV/IHNV-infected foci [17]. For the *in vivo* infections, supernatants from VHSV-infected cell monolayers were clarified by centrifugation at 4000 × g during 30 min and kept in aliquots at −70 °C. The spring viremia of carp virus SVCV isolate 56/70 [18] was grown in zebrafish embryonic fibroblast ZF4 cells [19] (ATCC, CRL-2050). ZF4 cells were maintained at 28 °C in a 5% CO_2 atmosphere in the same cell culture medium described above. To prepare SVCV, 2% FCS and 22 °C were used. To assay for inhibition of infectivity, SVCV was purified by pelleting SVCV-infected supernatants by ultracentrifugation at 60,000 *g* for 60 min at 4 °C and then titrated by methylcellulose plaque assays as described before [10].

2.2. Previously published experiments using zebrafish and its microarray data analysis

Zebrafish were maintained at 24–26 °C in 30 l aquaria with tap-dechlorinated carbon-filtered water with 1 g of $CaCl_2$, 1 g of $NaHCO_3$ and 0.5 g of Instant Ocean sea salts added to water resulting in a conductivity of 200–300 μS pH of 7.8–8.2, as described before [8–10]. The aquaria were provided with biological filters and fish fed with commercial flakes (Tetramin, Tetra GMBh, Germany). For each experiment, groups of 10 zebrafish were moved to 2 l miniaquaria provided with biological filters for acclimation during 7 days. Groups of 10 zebrafish were infected-by-immersion with VHSV or mock infected, as previously reported [8–10]. Briefly, zebrafish were maintained in 2 × 10^6 ffu of VHSV per ml at 14 °C during 2 h in 50 ml containers with continuous aeration and then released to their 2 l miniaquaria at 14 °C. After 2-days, fins (dorsal, ventral and caudal) or lymphoid organs (head kidney plus spleen) were harvested and independently pooled for 6 zebrafish per group, n = 4 groups per treatment. Extraction, labeling of high quality RNA and hybridization were performed as described before [8,10]. Differential expression folds of genes of the "complement and coagulation cascades" KEGG pathway were compared from hybridization data deposited in the Gene Expression Omnibus (GSE19049) [8]. Gene differential expressions or modulations expressed were calculated in folds by the formula, fluorescences of VHSV-infected zebrafish/mean fluorescences of non-infected zebrafish. Then the means ± standard deviations of the folds were calculated for n = 4. Genes with differential expression fold values > 1.5 or <0.66 were defined as statistically significant according to the one sample 2-tail independent t-test at $p < 0.05$. Other details of sample processing were as previously described in detail [8,10].

2.3. Design and synthesis of SERPIN-derived peptides

To specifically inhibit serine protease inhibitors (*serpin* genes, SERPIN proteins) we used SERPIN-derived peptides, since mammalian SERPINs are specifically inhibited by 14-mer peptides derived from their 28 amino acid (P14…P14′) Reactive Center Loops (RCL) [11,12]. To find the RCL sequences corresponding to zebrafish, P4.P3.P2.P1…P1'.P2'.P3'.P4'. amino acid sequences (one single letter amino acid codes around the scissile P1–P1′ bound) of each human SERPIN were retrieved [12] and aligned to the corresponding amino acid sequences in zebrafish. Orthologous peptide sequences corresponding to the 14-mer amino-terminal part of each zebrafish RCL SERPIN were then obtained from the P14…….P1 positions (Table 2). The 14-mer peptides were chemically synthetized by adding acetylation to its amino-terminal part as used before [11,12]. Cell penetrating peptide probabilities were estimated using predictive software at bioware.ucd.ie/CPPpred [24]. SERPIN-derived peptides and 4–7 amino acid subsequences of SERPINe1 (TKGSSAT, AAVIYSR, TKGS, SATA, and