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Establishment and characterization of a rainbow trout heart endothelial cell line with susceptibility to viral hemorrhagic septicemia virus (VHSV)

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ABSTRACT

In the current work, we have established and characterized a novel cell line from rainbow trout (*Oncorhynchus mykiss*). The cell line, designated as RTH (rainbow trout heart), was obtained by immortalizing heart cells with recombinant retroviruses that transduced polyoma middle T antigen. This is the first time such a strategy is used to obtain an immortalized fish cell line. The cells showed an endothelial-like morphology and characteristics, constitutively transcribing collagen, selectin and VCAM (vascular cell adhesion molecule), as well as different chemokines and chemokine receptors, but not cytokeratin. As already described for heart endothelial cells, RTH cells actively phagocytized latex beads. Furthermore, RTH cells showed a high susceptibility to viral hemorrhagic septicemia virus (VHSV). VHSV modulated the transcription of Mx, major histocompatibility complex II (MHC-II), VCAM and many of the chemokine and chemokine receptors expressed in these cells. Therefore, RTH cells constitute an excellent model to study the immune regulation of endothelial cells in fish and their role in leukocyte extravasation.

1. Introduction

In vitro models are highly valuable tools to directly study specific cellular mechanisms without individual variability. Because primary cultures are usually short-lived, easily contaminated and require specific culture conditions, the establishment of immortalized cell lines is a valuable research instrument to understand the physiology of a specific tissue.

To date, different cell lines have been established in rainbow trout (*Onchorhynchus mykiss*). The RTG-2 cell line derived from rainbow trout gonad was established in 1962 as a fibroblast cell line widely used with a broad range of viral susceptibility [1]. The RTS11, established in 1998 from rainbow trout spleen cultures, is a monocyte-macrophage cell line [2]. This cell line has been very useful to study different aspects of monocyte-macrophage functionality and their response to different pathogens [3–5]. RTS34 is another cell line obtained from a long-term spleen hemopoietic culture. This cell line consisted in a mixed stromal cell layer with an associated cell population of macrophage-like cells that form proliferative foci and release non-adherent progeny cells into the culture medium [6]. RTgill-W1, an epithelial cell line established from rainbow trout gill, has also been proposed as an important tool to study aquatic toxicology [7]. Up to date, however, no endothelial cell lines have been established to our knowledge neither in rainbow trout nor in other fish species.

In the current study, we have established an immortalized heart endothelial cell line from rainbow trout using supernatants from packaging cells producing recombinant retroviruses that transduced polyoma middle T antigen. Middle T antigen is a membrane bound polypeptide that interacts with a number of proteins used by tyrosine kinase associated receptors to stimulate mitogenesis [8]. This method has been very well accepted for the generation of cell lines in mammals, usually generating immortalized endothelial cultures [9–12]. In our case, the cell line obtained, designated as RTH (rainbow trout heart) also showed an endothelial-morphology and accordingly expressed collagen and the cell adhesion molecules selectin and VCAM (vascular cell adhesion molecule), but not

Abbreviations: HSMI, heart and skeletal muscle inflammation; IPNV, infectious pancreatic necrosis virus; ISAV, infectious salmon anemia virus; IFN-γ, interferon γ; MHC-II, major histocompatibility complex II; PRV, piscine orthoreovirus; RTH, rainbow trout heart; SPDV, salmon pancreas disease virus; TNF-α, tumor necrosis factor α; VCAM, vascular cell adhesion molecule; VHSV, viral hemorrhagic septicemia virus.

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cytokeratin. As reported for endothelial cells [13], and specifically for fish heart endothelial cells [14,15], RTH cells actively phagocytized latex beads. Because chemokines play an important role in the regulation of endothelial cell function, including proliferation, migration and differentiation during angiogenesis, and re-endothelialization after injury [16], we also studied their capacity to express rainbow trout chemokines and chemokine receptors.

In addition, the heart is an important target for viral infections in fish and constitutes one of the main replication sites for viruses such as viral hemorrhagic septicemia virus (VHSV) [17], infectious hematopoietic necrosis virus (IHNV) [18], the piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) [19] or salmon pancreas disease virus (SPDV) [20]. On the other hand, infectious salmon anemia virus (ISAV) specifically targets heart endothelial cells in salmon [21]. Therefore, the susceptibility of RTH cells to common viral pathogens from rainbow trout such VHSV or infectious pancreatic necrosis virus (IPNV) was also addressed in the current study. This newly established RTH cell line constitutes a novel tool to study the immune regulation of heart endothelium in fish and can be further used as a model in angiogenesis, homeostasis and leukocyte transmigration in fish as well as in VHSV pathogenesis studies.

2. Materials and methods

2.1. Generation of RTH cell line

Hearts were aseptically removed from rainbow trout (*O. mykiss*) of 10–12 cm under a dissecting microscope. Tissues from 2 to 3 animals were placed in tubes containing 7 ml of L-15 medium (Gibco, USA) and 0.15 mg/ml collagenase (Sigma). Single cell suspensions were accomplished by mechanical tissue dissociation followed by enzymatic digestion. Organs from 2 to 3 animals were placed on a sterile 100 mm tissue culture plate and minced using sharp scissors followed by scalpel blades into pieces as small as possible to increase the final cell yield. With a sterile spatula, the minced tissues were transferred to tubes containing 7 ml of L-15 medium (Gibco, USA) and 0.15 mg/ml collagenase (Sigma) and incubated for 30 min at 20 °C with agitation. The reaction was stopped by adding 7 ml of L-15 supplemented with 10% fetal bovine serum (FBS, Gibco). The non-digested tissue was discarded by retention on a 70 μm filter. Dissociated cells were washed by centrifugation and incubated on 0.1% gelatin-coated plates at 20 °C with L-15 supplemented with 20% FBS, 2% heparin (Sigma), 0.005% Brain and Heart Infusion broth (Merck), 100 I.U./ml penicillin and 100 μg/ml streptomycin (P/S). Non-adhered cells were removed after 2 h in culture. The next day, culture medium was replaced by medium without heparin.

The RTH line was established using a modified version from previous reports [11,12]. Subconfluent primary cultures (48 h post-seeding) were incubated in the presence of polybrene (8 μg/ml, Sigma) with supernatant from packaging cells producing recombinant retroviruses that transduced polyoma middle T antigen (virus concentration about 10–200 pfu/cell). The virus-containing medium was replaced the next day with fresh complete medium. Cells were subjected to a second round of infection after 2 days of incubation at 20 °C. Cells were passaged when confluent in L-15 supplemented with 20% FBS, 0.005% Brain and Heart Infusion broth and P/S. Non-infected cells died within a few passages.

2.2. Cell cultures

Following immortalization, after 10 passages in L-15 supplemented with 20% FBS, 0.005% Brain and Heart Infusion broth and P/S, RTH proliferating cells were maintained in L-15 supplemented with 10%FCS and antibiotics (P/S). Cells were grown at 20 °C and passaged at a 1:2 ratio every week using a 0.25% trypsin solution.

The epithelioma papillosum cyprini (EPC) cell line from fathead minnow (*Pimephales promelas*) was also used in this study. EPC cells were also cultured at 20 °C in L-15 supplemented with 10%FCS and antibiotics. Cells were grown at 20 °C and passaged at a 1:3 ratio every week.

2.3. RNA extraction and cDNA preparation

Total RNA was extracted from cells using a combination of Trizol (Invitrogen) and RNAeasy Mini kit (Qiagen). In summary,

Table 1
Primers used for real time PCR in this study.

Gene	Name	Sequence (5′-3′)
Collagen	Collagen-F	TCTCAACCCTTTTCTATGCATTTTTT
	Collagen-R	TTTAAGCAAGTGGACCATTTTTGAA
Selectin	Selectin-F	CTCATGTTACCATGGAGAGTGTGTG
	Selectin-R	CTCCTCCATCTTACATTCAACAACG
VCAM	VCAM-F	TCACTGTATGCTGTCGCTCTGTTAG
	VCAM-R	ACCTGGTATAGGCCTGTATCTTTGG
Cytokeratin	Cytokeratin-F	GGGTGGTTTGACCTCCTTAGCT
	Cytokeratin-R	CAAAGTGCATCCAGTAATTCCTATCA
MHC-II	MHCII-F	ACACCCTTATCTGCCACGTC
	MHCII-R	TCTGGGGTGAAGCTCAGACT
Mx	Mx-F	AGCTCAAACGCCTGATGAAG
	Mx-R	CTGGGTCCACAGTGTACATTTAGTT
N VHSV	N VHSV-F	GAGAGAACTGGCCCTGACTG
	N-VHSV-R	CCCGAGTTTCTTGGTGATGT
CK1	CK1-F	GATGGCTGAAAGGCTACACC
	CK1-R	TGGGATTTGTTCTCCTGACG
CK3	CK3-F	AGATCACCGTTCCCATCATC
	CK3-R	GTGACTTTCTGGCCATCTCC
CK5B	CK5B-F	TTTGCTGATCGTCAGATACCC
	CK5B-R	GTGTCTGCTCCCCAGACTTC
CK6	CK6-F	TGAAAGGCCTACGAATCTGC
	CK6-R	GTTGTTGTTGGCTGGTTGTG
CK7A	CK7A-F	CCGAGAATCCCTCTTCAACA
	CK7A-R	TCATCGTCGTCTTGGCAGTA
CK9	CK9-F	GGCTCTTATGGGAACTGCTG
	CK9-R	CTGGGATTGGCACAAACAG
CK10	CK10-F	ATTGCCAAGATCCTCTTCTGTGTTC
	CK10-R	CCTGAGGCTGGTAACCTATGACAAC
CK11	CK11-F	CCTTTGAGCATACTAATGCGAGTGG
	CK11-R	GTCTGCACAATACTTCCTCCCATTG
CK12	CK12-F	GACATCGATGCCACTGTGTT
	CK12-R	GGAGATGGTTCGCTCCAGAC
CXCL8_L1	CXCL8-F	ATTGAGACGGAAAGCAGACG
	CXCL8-R	CTTGCTCAGAGTGGCAATGA
CXCd	CXCd-F	GCTCACACTGCTCTAAGGAAGAA
	CXCd-R	GGAGAGAGTCTCAATGGAACGT
CXCL11_L1	CXCL11-R	TGGACTGGTGAACCGTGTTA
	CXCL11-F	TCTTGGCAAATGGAGCTTCT
CCR6	CCR6-F	TGCAGAGGAAACAGTTAACAATTCACC
	CCR6-R	CCAGTAAACCCAGGATACAGATGAC
CCR7	CCR7-F	TTCACTGATTACCCCACAGACAATA
	CCR7-R	AAGCAGATGAGGGAGTAAAAGGTG
CCR9	CCR9-F	TCAATCCCTTCCTGTATGTGTTTGT
	CCR9-R	GTCCGTGTCTGACATAACTGAGGAG
CR9B	CCR9B-F	AATATTTCCAACGTCTGAAACAGGA
	CCR9B-R	CTCACCCAGGACTTATCACACATTC
CCR13	CCR13-F	GTTCTGTACAACGTCTGGAAGGATT
	CCR13-R	ATGGCCAAAGGAAGTAGAAAGAAGA
CXCR1	CXCR1-F	CCTGATATCCAGAAGCTCTTTGTGT
	CXCR1-R	TTGCATCCAGCTCTATGATAATGAA
CXCR3A	CXCR3A-F	CAAGGCAACCACAAATTACTATATTTATGATG
	CXCR3A-R	CAGCACACACAGCACCAGGAT
CXCR3B	CXCR3B-F	CACTGGAGCCATGTTTACAATCAACT
	CXCR3B-R	CCCTCACAGACTCCAGGAAGTG
CXCR4	CXCR4-F	GTGCATGTGATCTACACCATC
	CXCR4-R	GAGCTGTGGCAAACACTATGT
EF-1α	EF-1α-F	GATCCAGAAGGAGGTCACCA
	EF-1α-R	TTACGTTCGACCTTCCATCC