New Phenomenon

**Lamprey serum can kill HeLa and NB4 tumor cells**

Yinglun Han1,2†, Yue Pang1,2†, Tao Yu1,2, Rong Xiao1,2, Biyue Shi1,2, Peng Su1,2, Xin Liu2, and Qingwei Li1,2*

1College of Life Science, Liaoning Normal University, Dalian 116029, China
2Lamprey Research Center, Liaoning Normal University, Dalian 116029, China
†These authors contributed equally to this work.
*Correspondence address. Tel: +86-411-85827799; Fax: +86-411-85827799; E-mail: liqw@263.net

Nature consists primarily of living materials, many of which are pathogenic microorganisms capable of killing and converting other organisms into copies of themselves [1,2]. For self-defense, eukaryotes have gradually evolved immune systems, including the innate and adaptive immune systems [3–7]. Innate immunity has been found in plants, fungi, and metazoans; while jawed vertebrates have an evolved adaptive immune system in addition to the innate immunity defense [8]. Agnathans, represented by lamprey [9] and hagfish, are the oldest vertebrates that possess the adaptive immune defenses, although whether TCR and BCR exist and VLRs are expressed in lymphocytes that resemble T cells and B cells of jawed vertebrates, respectively. After being infected by a specific pathogen, B-cell-like lymphocytes can kill HeLa and NB4 cells in vitro. The phenomenon of cell killing in lamprey is different from the traditional cytotoxic effect in jawed vertebrates, and the results might be helpful for the research on the early diagnosis and therapy of neoplastic diseases of human beings.

Adult lampreys were collected from the Tongjiang Section of the Heilongjiang River (Tongjiang, China) and housed in sand-lined aquaria at 20°C. HeLa and NB4 cells were supplied by College of Life Science, Liaoning Normal University (Dalian, China). HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St Louis, USA) and NB4 cells were maintained in RPMI 1640 medium (Sigma-Aldrich). Both media were supplemented with 10% fetus bovine serum (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), and 100 μg/ml streptomycin (Sigma-Aldrich). Cells were cultured in an incubator humidified with 5% CO2 and 95% air at 37°C. Normal adult lamprey (200–220 g in weight) was tail-severed, and blood was drawn into a 10-ml plastic centrifuge tube and allowed to clot at 4°C overnight. Serum was separated by centrifugation (1680 g) for 10 min at 4°C and was stored in a 1.5-ml centrifuge tube at −20°C. Before use, 5 ml of lamprey serum was dialyzed (molecular weight cut-off, 10 kDa) against phosphate buffered saline (PBS, three changes, a total of 2 l) for 24 h and adjusted to a final protein concentration of 20 mg/ml.

Cell death analyses were performed using propidium iodide (PI, Sigma-Aldrich) staining with subsequent fluorescence-activated cell sorting (FACS) analysis. HeLa and NB4 cells (5 × 10⁶) were incubated with 20 mg/ml lamprey serum dialyze for 15 min at room temperature, and PBS was used as a negative control. After that, the cell cultures were centrifuged at 150 g for 5 min, and the cells were collected, washed, and resuspended in 1 ml of cold PBS. The cells were further incubated with 100 μl of PI for 15 min at room temperature, and then 400 μl of 1 × annexin-binding buffer was added for flow cytometry (FACS Aria II; BD Corporation, New York, USA) analysis. The results showed that incubation of lamprey serum with HeLa and NB4 cells for 15 min caused outer-membrane disruption and led to the formation of blebs (Fig. 1A). At this time, the membrane quality was assessed by monitoring the membrane integrity. There was a higher percentage of cell death for HeLa (98.3%) and NB4 (96.2%) cells incubated with lamprey serum than for HeLa (18.8%) and NB4 (17.6%) cells incubated with PBS (Fig. 1B). Most cell death occurred within a 15-min incubation in lamprey serum (Fig. 2, P < 0.01).

For morphological studies on cell death, HeLa and NB4 cell suspensions (5 × 10⁶ cells/ml in PBS) were centrifuged at 150 g for 5 min, and then cells were collected and resuspended in 200 μl of 20 mg/ml lamprey serum dialyze (PBS as negative control) at room temperature for 15 min. After that, the cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in PBS for 2 h at room temperature and...
then dehydrated in a graded series of ethanol. The cell samples were subject to critical-point drying using liquid CO₂ and coated with platinum using a sputter coater, and then photographed using a field emission scanning electron microscope (KYKY-1000B SEM; KYKY Technology Development Ltd, Beijing, China). SEM analysis of the structural morphological features showed that the tumor cells treated with PBS were intact and possessed a smooth surface with a clear globular shape, whereas cells treated with the lamprey serum showed damage of the membrane and leakage of cellular components. Cell damage to the outer membrane of HeLa cells was observed by SEM following incubation with lamprey serum, and the result showed that a pore was formed at the surface of a HeLa cell (Fig. 3A), with diameter of the pore-like structure >1 μm. As for NB4 cells, the initial morphological alterations of death occurred in the cell membrane (Fig. 3B), and some prominences and channels were formed at the surface of the membrane.

Figure 1. The effect of lamprey serum on cell death of HeLa and NB4 cells After culture under lamprey serum at room temperature for 15 min, HeLa and NB4 cell death were analyzed. (A) Microscopy analysis of cell death induced by lamprey serum. Blebs are indicated by black arrows. Magnification: ×500. (B) FACS analysis of cell death induced by lamprey serum. The percentage of HeLa cell death in lamprey serum (98.3%) was higher than that in PBS (18.8%). Similar results are shown for NB4 cells, in which the percentage of NB4 cell death in lamprey serum (96.2%) was higher than that in PBS (17.6%). Positive PI staining represents the dead cells.

Figure 2. The statistical analysis of the effects of lamprey serum on cell death of HeLa and NB4 cells The effects of lamprey serum on cell death of HeLa and NB4 cells were analyzed statistically. Data are presented as mean ± SEM of three independent experiments. **P < 0.01.

Figure 3. The morphological analysis on the cell death effect of lamprey serum on HeLa and NB4 cells (A) Morphology of dead HeLa cells in late phases after incubation with lamprey serum at room temperature for 15 min. Pore-like structure was formed on the dead cell membrane. Scale bar: 2.5 μm. (B) NB4 cells at various stages of cell death process. The cell in the bottom of the photomicrograph showed a normal morphology, the cell in the center showed degeneration, and the one at the top of the picture exhibited a late phase of death. Scale bar: 10 μm.
The cytolysis effect of lamprey serum on tumor cells was also analyzed by TEM. HeLa and NB4 cells ($5 \times 10^5$ cells/ml in PBS) were suspended in 200 μl of 20 mg/ml lamprey serum dialyzate (PBS as negative control) and incubated at room temperature for 15 min. After that, the cells were fixed with 1% osmium tetroxide (Sigma-Aldrich) for 2 h. The cells were then washed with PBS and dehydrated in an ethanol series (25%, 50%, 75%, 95%, and 100%), transferred into propylene oxide, and embedded in Epon812. Ultrathin sections were obtained with a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzla, Germany) and mounted on a formvar-coated brass grid. The sections were stained with 2% (w/v) uranyl acetate in 70% (v/v) methanol and 0.5% lead citrate. Observations and image recording of the cells were performed with a JEM-2000EX TEM (JEOL Ltd, Tokyo, Japan). TEM examination of the cytolysis of tumor cells showed that, after incubation with serum, the cells underwent a rapid necrosis process and exhibited swelling and loss of membrane integrity. The nucleus swelled obviously, which almost filled the whole cell (Fig. 4A,B). Furthermore, the number of free ribosomes increased, which finally led to lysis of the target cells. These results affirmed that the lamprey serum has anti-tumor cell activity, which is accomplished through membrane breakage.

In this study, we found that lamprey serum possessed important cytotoxic effects on tumor cells. After a 15-min incubation with lamprey serum, the morphological changes and cell organelle damage of the HeLa and NB4 cells could be observed. As for HeLa cells, a pore-like structure was formed on the membrane with the diameter being $\sim 2 \mu m$, which is much bigger than the pore of terminal complement complex of complement ($\sim 10 \text{ nm}$). As for NB4 cells, several cell protuberances were formed on the surfaces of NB4 cells, which finally led to the leakage of cytoplasm. These results indicated that lamprey serum could induce the disruption of outer membrane, and the release of cell contents into the external environment. These features indicated that lamprey serum has an important influence on tumor cell membrane systems which contain plasma membrane, nuclear envelope, and mitochondrial outer membrane, and finally results in tumor cell lyses. Therefore, the cell death process induced by lamprey serum was quick, violent, and irreversible. This novel phenomenon of cell killing induced by lamprey serum is different from the serum cytotoxicity of jawed vertebrate. Jawed vertebrate serum cytotoxicity is always mediated by complement system. The destruction process of complement system only causes outer-membrane disruption and leads to the formation of blebs. However, the inner membrane and organelles remain intact. At the same time, a complement system does not cause formation of cell protuberance at target cell surface [12–14]. The character of

![Figure 4](http://dx.doi.org/10.1093/abbs/ggt120)
normal lamprey serum killing may prove that some unknown functional proteins possibly participate in the innate immune response and serve as in recognition of various pathogens in this oldest agnatha. In the future research, we are going to isolate and purify function proteins which could be involved in lamprey serum cytotoxicity, as well as to further elucidate the mechanism using in vivo tumor models.

**Funding**

This work was supported by the grants from National Program on Key Basic Research Project (973 Program) (2013CB835304), National Natural Science Foundation of China (General Program) (31170353, 31271323), National Science Fund for Distinguished Young Scholars (3122020, 31301880), and Natural Science Foundation of Liaoning Province of China (201202120).

**References**