Developmental and Comparative Immunology

Short communication

The primitive immune system of amphioxus provides insights into the ancestral structure of the vertebrate immune system

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1. Introduction

Amphioxus (the lancelet, subphylum Cephalochordata), which is the contemporary representative of an ancient chordate lineage with a fossil record dating back to the Cambrian period, is considered to be the basal chordate (Putnam et al., 2008; Holland et al., 2008). Together with vertebrates and urochordates, amphioxus descended from a common ancestor that lived around 550 million years ago (Putnam et al., 2008). In many respects, amphioxus reflects the primitive vertebrate condition, but also exhibits uniquely specialized features that arose in the half billion years since its divergence from the main chordate lineage (Holland et al., 2008). Recent studies placed urochordates as the sister group of vertebrates (Blair and Hedges, 2005; Delsuc et al., 2006), and revealed that amphioxus was not a close relative of vertebrates as had previously been thought (Putnam et al., 2008; Huang et al., 2008), which only increased its importance in our understanding of fundamental features of the chordate ancestral condition.

Amphioxus is widely used as a model for studying evolution and development and there are extensive observations on its locomotor, sensory, and nervous systems (Holland et al., 2004). However, very few studies of its immune system have been conducted, especially of the structure and evolutionary significance of immune tissues and organs.

In Elie Metchnikoff’s late 19th century study of amphioxus, which he called the last survivor of the lower vertebrates, he stated “all attempts therefore to provoke inflammatory phenomena in it have given only negative results” (Metchnikoff, 1891). For many years, it was not known how amphioxus could survive microbial infection. However, two decades ago, Rhodes et al. (1982) reported a small number of free and fixed macrophage-like cells were also found in the amphioxus gut. The current results described the structure of the immune system and cellular defense against infection in a protochordate, which may help us in understanding the structural origin of the vertebrate immune system.

Keywords: Amphioxus, Vertebrate, Immune system, Structure, Gill, Gut

Abbreviations: TNFR, tumor necrosis factor receptor; S.c., Staphylococcus aureus; V.p., Vibrio parahaemolyticus.

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pensable role in immune defense against microbial challenge (Liu et al., 2009). Gerzeli (1961) reported the presence of enterochromaffin cells in the gut of amphioxus but did not identify their function or the basis for their role in defending against certain infection.

We have been striving to study the immune system of this animal at the molecular level for many years. This has led to some important findings such as the galectin (Yu et al., 2007a) and the C-type lectin system (Yu et al., 2007b), the tumor necrosis factors system (Yuan et al., 2007), the V and C domain-bearing protein (Yu et al., 2005), toll like receptors (Yuan et al., 2009a), tumor necrosis receptor associated factors (Yuan et al., 2009b), and especially lymphocyte-like cells (Huang et al., 2007a). These results contribute to a better understanding of the amphioxus immune system. A systematic comparative analysis of the amphioxus genome database was also carried out in our laboratory (Huang et al., 2008), which showed that it has an innate immune diversity and complexity unparalleled among the vertebrates. However, there has been no systematic study of the anatomy of its immune system.

In this study, we try to reveal the structure of amphioxus gut and gill and offer some cellular evidence of its immune defense system. We also want to further understand the relationship between the structure and function of this primitive immune system and to see whether amphioxus has the same inflammatory response as that in vertebrates, and then to compare the structural similarities and differences between amphioxus and vertebrates.

2. Materials and methods

2.1. Amphioxus care and maintenance

Mature adults of Chinese amphioxus, Branchiostoma belcheri tsingtaunese, were obtained from Kioachow Bay near Qingdao, China, and cultured in our laboratory in aquaria supplied with circulating filtered seawater maintained at 20–25 °C.

2.2. Immune challenge of animals

For light microscopy, live Staphylococcus aureus (S.c) was suspended in PBS and 0.5 µl (10⁶ cells/ml) was microinjected into muscle that located at one-third of anterior back of each individual amphioxus. Amphioxus which were microinjected with 0.5 µl PBS were used as controls. We use 30 amphioxus for experiment group and 30 for control group. The animals were kept in the aquaria for 5 days.

For electron microscopy, a total of 5 µl (10⁶ cells/ml) of live Vibrio parahaemolyticus (V.p) in PBS was microinjected into the position of one-third of hindgut from the anus of each individual amphioxus. Amphioxus which were microinjected with 5 µl PBS were used as controls. We use 24 amphioxus for experiment group and 24 for the control group. The animals were kept in the aquaria for 2 days.

2.3. Microscopy

For light microscopy, the whole body of S.c injected amphioxus were fixed in 4% formaldehyde. Fixed tissue was washed with PBS, dehydrated in a graded series of ethanol, embedded in paraffin, cut in 5 µm sections and stained with hematoxylin and eosin. Amphioxus injected with PBS only was used as controls.

For electron microscopy, at the time point of 6, 12, 24 and 48 h after the V.p. injection, the amphioxus guts were dissected under the microscope and cut into small pieces. The cut samples were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, and washed with PBS. After dehydrating in a graded series of ethanol and acetone, the samples were embedded in fresh Spurr’s Resin (Spurr embedding kit, EMS). Ultrathin sections were stained with uranyl acetate and lead citrate for examination. Amphioxus injected with PBS only were used as controls. Images were collected on JEOL 100CX-II at 100 kV.

2.4. In situ analysis of amphioxus tumor necrosis factor receptor 1 (named as BbtTNFR1)

Adults of the Chinese amphioxus Branchiostoma belcheri tsingtaunese, were collected and kept in filtered seawater for 2 days. The animals were sacrificed and cut at 1-cm intervals. The tissue blocks obtained were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Tissue blocks were cut transversally and mounted on glass slides coated with poly-lysine. The digoxigenin-labeled probes were prepared by using the plasmids that contain the sequences of the BbtTNFR1 with the SP6 promoter sequence at the 3’ end of the sequences as template and the antisense probes were synthesized with the SP6 RNA polymerase according to the protocol of the digoxigenin DIG RNA labeling kit (Roche). In situ hybridization was performed according to Li et al. (2004) with a little modification. That is before hybridization, the sections were de-waxed, re-hydrated, abstered, bleached with 3% H2O2 to inhibit endogenous enzyme activity, and digested by proteinase K. The probes were added to the sections at a concentration of 1 µg/ml. After overnight hybridization at 42 °C, sections were treated with a series of high stringency washes followed by immunodetection.

3. Results and discussion

3.1. Histopathology of amphioxus muscle following microbial challenge

To detect whether amphioxus has the same inflammatory response as that in vertebrates, S.c. were injected into the amphioxus muscle and the histopathological change were observed. Compared with PBS injection group, which had shown no obvious damage; after 24 h, the S.c. injected amphioxus swam erratically and 96.6% of amphioxus showed redness of the surrounding injection site. The histopathological change of the injection site was further studied under light microscopy. The phenomena of necrosis in the site of injection with normal muscle structure lost were found (Fig. 1A and B). However, no cells with the characteristics of phagocytes were seen infiltrating the lesions or phagocytizing necrotic tissue (Fig. 1C), which further confirmed the previous observations and investigations by Metchnikoff (1891) and Silva et al. (1995) regarding the absence of phagocytes in a wound and lack of inflammatory response in amphioxus. The potential molecular mechanism behind this observation may be due to the over-expressed protease cascade and protease inhibitory components (Huang et al., 2007b), which contributed to the digestion of the necrotic tissue after wounding or infection. This was obviously different from mechanisms seen in invertebrates which mainly use phagocytes to perform this function. The lesions in 90% S.c. injected amphioxus did not heal, but slowly progressed, leading to its death, indicating that the clearance of extruded microbes by enzymatic mechanisms might not be under effective regulation in most amphioxus. Surprisingly, we found that about 10% of amphioxus could survive and have tissue repair (Fig. 1D) after 5 days injection compared to the control group which were injected with PBS. We further analyzed the process of the tissue repair and found that during the early stages of repair, there is a granulation tissue-like substance that is later replaced by fibrosis, which fills into the space cleared of necrotic tissue. Thus, it seems that the tissue repair ability in amphioxus was limited and showed individual differences.
3.2. The putative lymphoid-like tissues in amphioxus gill

In our previous study (Huang et al., 2007a), we reported that under the epithelium near the basement membrane in the gill, some immature lymphocyte-like cells with apparently less electron-dense nuclei could be seen. In the current study, we further studied the lymphoid-like tissues in amphioxus gills. We found that the gill was composed of gill bars, which were formed of a core of muscle with an outer sheath of epithelium (Fig. 2A). In electron microscope sections, clusters of lymphocyte-like cells with large nuclei surrounded by a thin layer of cytoplasm were seen in the epithelium of the gill (Fig. 2B and C). The nuclei contained a peripheral rim of heterochromatin adjacent to the nuclear envelope. Unexpectedly, a lymph node-like structure, which may be the primitive form of the vertebrate lymph node and is believed to be one of the important components of the lymphoid system, was also seen in the epithelium (Fig. 2D).

3.3. Structural similarities and differences of gut between amphioxus and vertebrate

In the vertebrate gut, the absorptive cell and the goblet cells in the mucous membrane contribute to the digestion and absorption of nutrients. Paneth’s cell and the lymph nodes in the lamina propria mainly contribute to the immune defense against microbes. The structure of the amphioxus gut shares certain similarities with...
Fig. 3. The structure of the guts under the light and transmission electron microscopy. (A) The structure of muscle and gut under the light microscopy. Scale bar, 50 μm. (B) The apical surfaces of the cells formed cell protrusions that pinched off and entered the gut lumen. Scale bar, 2 μm. (C) The mitochondria in column-like epithelium. Scale bar, 1 μm. (D) The development of cell protrusions in apical surfaces of columnar epithelium. Scale bar, 3 μm. (E) The villus channel in the basement membrane. Scale bar, 2 μm. (F) Blood vessel in the gut of PBS control group. Scale bar, 2 μm. (H) When amphioxus was challenged with the V.p, many phagocytes, which contained the phagosome, were found in the vessels. Scale bar, 2 μm. The picture showed muscle (M), mitochondria (Mi), cell protrusions (CP), column-like epithelium (*), secretory granule (S), basement membrane (BM), villus channel (VC), blood vessel (BV), endothelial cell (EC), lysosome (Ly), phagocyte (Ph).

The vertebrate's such as the absorptive cell and the goblet cell in the mucous membrane, but the boundary between the mucous membrane and the submucosa was not apparent in amphioxus. The anterior mid-gut was lined with a tall column-like epithelium. The apical surfaces of the cells, which enveloped some secretory granules, formed cell protrusions that pinched off and entered the gut lumen (Fig. 3B). Many mitochondria in column-like epithelium could be seen, and this may be closely related to the energetic metabolism of epithelia whose metabolic process needed a large amount of energy (Fig. 3C). The secretory granules accumulated in the protrusion, and slowly progressed, leading to the disconnection with epithelium. The protrusion swelled, and dissolved, then died with releasing the secretory granules and other contents (Fig. 3D).

Unlike the urochordate colonial ascidian Botryllus schlosseri (Burighel and Milanesi, 1977), the amphioxus gut lacks microvilli in the epithelia. Moreover, there is no muscular layer in amphioxus gut which mainly participates in the movements of the enteron when compared with vertebrate. The amphioxus also has a thicker basement membrane and the villus channel in the basement membrane is bigger (Fig. 3E). We also found that the amphioxus has a large visible blood vessel in the gut and there are endothelial cells which contain many lysosomes lining in the blood vessel (Fig. 3F). Compared with amphioxus of the PBS control group, when amphioxus was challenged with the V.p., many phagocytes containing a large phagosome were found in the vessels (Fig. 3G and H), which were obviously different from the vertebrates. Furthermore, at the molecular level, amphioxus tumor necrosis factor receptor 1 (named as BbtTNFR1), which is just one of the important immune-related genes, was expressed in gut. Results of in situ analysis showed that the mRNA of BbtTNFR1 could be detected in the intestines and sexual-gland (Supplementary Fig. 1). The immune-
related genes localizing in gut may indicate an important role of the gut in defending against pathogen infection.

Given the evolutionary significance of this animal, all these differences indicate that each organ in amphioxus might have various functions, although it has more simple tissue organization than vertebrates, but the function of homologous organs became more specific when they evolved into vertebrates during evolution.

3.4. The phagocytosis phenomenon in amphioxus gut

The appropriate distribution of immune cells among organs in the body is crucial for the immune surveillance and effector functions of the immune system (Moser and Loetscher, 2001). This is also true for amphioxus. When V.p. was injected into the gut of amphioxus, after 48 h, many cells were seen to penetrate into the gut. In order to further understand the characteristics of those exudative cells, we used electron microscopy to observe the structural changes of the gut. Contrary to the amphioxus of the PBS control group which has normal epithelial cells with vacuoles and cilia of the amphioxus gut (Fig. 4A), many epithelial cells with large secondary-lysosome-like bodies were observed after the V.p. injection (Fig. 4B), and specially, those cells showed active phagocytosis.

A process of phagocytosis could also be seen in the gut of the infected amphioxus while compared with the PBS control group in which no obvious phagocytosis was seen. In the first step of phagocytosis, macrophage-like cells became attached to the V.p. (Fig. 4C) and encapsulated the V.p. through phagocytosis to form a phagosome. The phagosome fused with a lysosome to form a phagolysosome (Fig. 4D), which was substantiated by the finding of the genes related to energy metabolism (such as cytochrome c oxidase subunit) up-regulated by bacterial infection in our previous study (Huang et al., 2007b). Under the basement membrane, following infection with V.p., some phagocytes with lysosome-like bodies were also observed in the lamina propria (Fig. 4E and F).

Thus, the issue about “the existence of phagocytes in the amphioxus is a matter of debate” has been obviously resolved in this study. The occurrence of the functional macrophage-like cells in amphioxus suggests that a primitive form of vertebrate immune system is found in this animal during evolution. Moreover, the active phagocytosis of those epithelial cells indicates that besides the digestive and absorptive function for all epithelia, the epithelia of amphioxus gut might play an even more important role in the immune defensive function. The primitive immune cells may have originated from those ancestral cells which have been thought only as a specific function for digestion and absorption in the digestion system of vertebrates, suggesting that the immune system is originated from digestive system.

Thus, the current paper was very helpful for us to study the ancestral structure of the vertebrate immune system. Considering the fact that amphioxus hold so many distinct features compared with vertebrate, we might believe that the amphioxus will provide an insight to uncover novel defense mechanisms against infection, to further reveal new roles of phagocytes to understand its physiological and pathological roles in vertebrates. In addition, further functional analyses of those identified tissues and structure, along with the advances from the molecular biological studies from this animal will put the amphioxus in a key position to understand the origins and characteristics of vertebrate immune system.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.03.009.

References