Molecular characterisation of NKR-P1 receptor in peripheral blood of pig

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Abstract

Characterization of natural killer receptor-p1 (NKR-P1) in the leukocytes from peripheral blood of pig revealed three different clones of NKR-P1, 1,2 and 3. The RT-PCR product of human NKR-P1 gene was 573 base pair (bp). The RT-PCR product of pig NKR-P1 gene was 661bp.

Keywords: Molecular characterisation, NKR-P1 receptor, Blood, Pig, Natural Killer Cell.

Introduction

Natural Killer Cells (NK-Cells): - Natural killer cells are a lineage of large granular lymphocytes. They are a sub-population of lymphoid cells that are present in most normal individuals of a range of mammalian and avian species (Hebermann and Ortaldo,1981).

NK cells have activatory or inhibitory motifs in their cytoplasmic domain of their receptor. They are primarily marked by their opposing signals and can be differentiated basically on molecular and functional basis. They are able to recognize different groups of HLA class I molecules. There are human NK receptors characterized by an extracellular region and cytoplasmic domain of different length. Long cytoplasmic domain displays usual immune receptor tyrosine based activation (ITAM) motif. These NK receptors display a limited number of amino acid variants. They correlate with NK inhibition by group of class I HLA allele. So expression of NK inhibitory receptors with 2-immunoglobulin superfamily (IgSF) is corresponding to HLA-C alleles, whereas 3 IgSF domain is corresponding to HLA-B alleles (Dohring and Colonna, 1996).

NKR-P1 receptor (CD161): - There are three different types of NKR-P1 in rat and mice, namely NKR-P1A, NKR-P1B, and NKR-P1C. Only one homologous NKR-P1A has been found in human. It is located in chromosome 12 in human, chromosome 4 in rat, and chromosome 6 in mouse. In mouse it has conserved motif (CxCP) in cytoplasmic domain. But conserved motif lacks in human. Some of NKR-P1 has ITIM motif. They are seen in NKR-P1B isoforms in rat and mouse. So it can inhibit NK cell function rather than activate (Lanier, 1998).

Broadly NKR-P1 can be divided into 2 groups.

a) **C type lectin, transmembrane type II receptors:** ly-49, NKR-P1, CD-69, CD-94, NK G2 etc. In lectin types protein extracellular domain possess receptor, it may be calcium dependent.

b) Immunoglobulin type, natural killer receptor (NKR): it consists of natural killer cell inhibitory receptor family. Basically they display domains like immunoglobulin in extracellular region. The killer cell inhibitory receptor (KIR) has been localized on human chromosome 19q13.4. They encode structures typical of type I transmembrane (amino-terminus extracellular) molecules belonging to the IgSF (Steffens *et. al.,* 1998).

The CRD sequences are most highly conserved in C type lectin family. There are significant homologies in the cytoplasmic domain and the extracellular domain proximal to the transmembrane domain, suggesting that these genes probably originated from a common ancestral precursor (Wong et. Al., 1991)

NK 1.1 antigen might binds with ligands but lysis for formation of cytotoxicity does not carry out in the absence of calcium (Ryan *et. al,* 1992).

The CD 161 molecule recognizes carbohydrate

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structure in target cell and delivers stimulatory signal. The NKR-P1 molecule was recently identified as one of the important NK cell receptors and it recognizes certain kinds of oligosaccharides on target cells and triggers NK cells for cytotoxicity (Arase *et.al*, 1996).

The NKR-P1 is a 60 KDa homodimer expressed on all rat NK cells (Ryan *et. al,* 1994). Rodent T cells infrequently expresses NKR-P1. At first NKR-P1 was identified by the 3.2.3 Mab that recognizes a disulfide-linked homodimer expressed on rat NK cells, grnnulocytes and on a minor subset of T cells (Lanier *et. al,* 1994).

NKR-P1 belongs to the c type lectin family, there is calcium dependent ligand binding. The ligand is glycoprotein, c type lectins bind oligosaccharides with terminal galactose N-acetylglucosamine or mannose. The c type lectin family would have a role NKR-P1 Receptor in peripheral blood of pig in the control of cell numbers during haemopoiesis and transplantation reactions. They have stalk region, a cytoplasmic tail along with tyrosine or serine residue.

The antibodies bound to NKR-P1 on the surface of NK cells can induce antibody dependent cytotoxicity of FcR+ target cells and cross linking NKR-P1 with antibodies stimulates phosphoinositide turnover and mobilization of intracellular calcium (Benzouska et al, 1994). Virtually there is not much known about signal transmission via CD94 and NKR-P1. In this process NKR-P1 mediated signal pathway involves 1,4,5-triphophate whereas CD94 activates phospholipase D and calcium mobilization. NKR-P1 and ly-49 proteins have structural homology to known surface receptors including the hepatic asialoglycoprotein receptors and low avidity IgF receptor, FceRII. There is no similarity between cytoplasmic domain of ly-49 and NKR-P1. That is why NKR-P1 acts as activatory whereas ly-49 acts as inhibitory.

Objective

The objective of this work is the molecular characterization of the NKR-P1 receptor expressed on peripheral blood of NK cells in pig. Natural Killer (NK) cells are distinct lineage of a sub-population of large granular lymphocytes. NK cells contribute a vital role in immune system.

NKR-P1 is well expressed in peripheral blood of man. It is one of the markers for T and NK cells. Currently there is no pig NKR-P1 gene sequence available in a GenBank. So by aligning known sequences in the human rat and mouse, NKR-P1 homologous sequence can be determined. From which it is possible to design primer.

By extracting total RNA, cDN A can be obtained from reverse transcription. Human RNA could be taken as a positive control.

Material and Methods

Total RNA was extracted from the leukocytes collected from peripheral blood of pig and human. Reverse transcriptase PCR technology was used to yield the total RNA. The PCR product was cloned in pPCR- script TM Amp SK(+) cloning vector. The plasmid DNA was extracted and purified for sequencing reaction. Three different clones on NKR-P1 nucleotide sequence obtained were aligned with available sequences of NKR-P1 gene from GenBank.

Pig blood was collected from pig slaughterhouse in Ghent Veterinary College, Belgium. Chicken blood was collected from chicken in KUL, Leuven. I had donated blood for doing RT-PCR of human NKR-P1 gene.

Denaturation and annealing of primer was done by heating solution (RNA, reverse primer, DEPC water) at 80 ^{o}C for 3 minutes. Then it was immediately put on ice. Then freshly prepared RT-premix was added on each tube. AMV reverse transcriptase had been used. Reverse transcription is carried out for 45 minutes at 42 ^{o}C. Then sample was heated at 95 ^{o}C for 3 minutes. Finally it was cool down to 4 ^{o}C. Afterward freshly prepared PCR premix was added in each tube. The PCR cycle is as follows.

1. 93 ^{o}C for two minutes.
2. 93 ^{o}C for one minute.
3. 53 ^{o}C for one minute. It is annealing temperature.
4. 72 ^{o}C for one minute. It is extension temperature. Repeat cycle from 2-4 for 39 times.
5. 72 ^{o}C for five minutes.
6. 4 ^{o}C forever.

The reference sequence for NKR-P1 gene is as follows from GenBank.

M77678, M77677, M 77676, U11276, U56936, X64716, X64717, X64718, X64719, X64720, X64721, X64722, X64723, X64724 and X97477.

Primer used:

NKR-P1 R- 5' AGTTCCTTTTGGCAGATCCA3'

F- 5' ATGAGTCACCTCCATCTCTT3'

The blood was centrifuged at 2200 RPM for 30 minutes without break at 20 degree C. It was separated in6 tubes by Ficoll density gradients. Then the content was divided in 3 tubes of 20 ml each. It

was centrifuged at 18000 RPM for 20 minutes without break. 2-tubes were made by adding alsevers up to 50 ml. The content was centrifuged at 1200 RPM for 15 min.

Preparation of agarose gel and electrophoresis: 2% agarose gel was made. One gram of agarose was mixed with 50 ml of TAE 1% buffer. It was heated in microwave till dissolved completely. Then it was taken out. It was cool down for some time. Then 2 microgram of ethidium bromide was added on it. Then it was poured down slowly in the plate having comb. When it solidify, comb was taken out. The plate was put in the electrophoresis device, filled with TAE 1% buffer. After loading the samples, it was run for 45 minutes at 10 volts. Then gel can be observed in the ultraviolet ray for bands. If bands were traversed considerable distance, gel could be taken for photograph. The picture was taken in Image Master NKR-P1 Receptor in peripheral blood of pig VDS. It was set for the best picture, which we could see. Then picture can be saved for further processing and calculating molecular weight in computer.

Results

In peripheral blood of pig (PBP) there was 853-leukocyte/cu mm and total cells were $5x10^7$ cells/ml. 6aliquots were made each of 200ml. Each aliquot should contain $1x10^6$ cells/ml. Therefore, there was good yield of total RNA from RT-PCR.

In peripheral blood of human (PBH) there was 360 leukocytes/cumm and total cells were $1x10^7$ cells/ ml. 4 aliquots were made each of 200ml. Each aliquots was about $1x10^6$ cells/ml.

Peripheral blood of chicken (PBC) there was 461 leukocytes/cumm and total cells were $1x10^8$ cells/ml. 23 aliquots were made each of 200ml. Each aliquots was $1x10^6$ cells/ml.

Chicken spleen (CS) there was 362 leukocytes/cumm and total cells were $1x10^8$ cells/ml. 10 aliquots were made each of 200ml. Each aliquots was more than $1x10^6$ cells/ml.

In the beginning of the experiment there was low yield of RNA. It was due to Rnase contamination. There were evidences of faulty handling procedure. PCR product of PBH was 573 bp. PCR product of of PBP was 661 bp.

Discussion

NK cells are cytotoxic cells. They can kill or lyse virally infected cell, tumor cells or normal cells without prior sensitization. They can kill efficiently the cells losing their Major Histocompatibility (MHC) class I expression. There was no memory system detected in NK cells. They are not T- cell lymphocyte populations spontaneously mediating cytotoxicity (Biron, 1997).

NK Cells do not express the CD3 neither complex nor any of the known T cell receptor changes (TCR) (Trinchieri, 1989). Freshly isolated NK cells express zeta chain of CD3/TCR complex (Yokoyama and Seaman, 1993).

NK cells are effector cells in a defense system geared to detect the deleted or reduced expression of self-MHC (karre *et. al*, 1986).

The interaction between TCR and MHC class I molecule activates cytolytic function whereas interaction with NK receptors inhibit cytolytic function (Cantoni *et al*), 1996).

Karre's "missing self" hypothesis states that the NK cells will detect and eliminate cells, which do not express MHC, class I molecules. Some cells may escape T cell recognition, but can be killed efficiently by NK cells. NK cells play important role in cell-mediated immune response.

By the help of antibodies produced by the humoral immune system, the cellular immune system can stimulate NK cells and cells of the nonspecific defense system to kill targeted cells. This process is called as antibody dependent cell mediated cytotoxicity (ADCC) (Tortora et. al, 1995). Activatory NK cell receptors include CD2, CD16, CD28, CD161, (Lanier, 1998) and NKG-2 (Ref).

Non activatory NK cells receptors are CD18, CD35, CD39, CD56, CD94, Natural killer cell inhibitory receptor (KIR), KIR103, NK receptors for HLA-C alleles, Immunoglobulin like transcript 1 and 2(Ref).

NK cell receptors contain ITIAM (I/V xYxxL/V) sequences in the cytoplasmic domain except CD94 (Lanier, 1998). Monoclonal antibodies (mAb) against mouse and rat NKR-P1 trigger pphophoinositol and arachidonic acid production, which will increase calcium level and result in NK cell activation for cytotoxicity and cytokine production (Lanier, 1998). The NKR-P1 and ly-49 proteins are only distantly related to the NKG2 proteins and cDNA sequences have no significant similarity to the NKG2 transcripts (Houchins *et. al*, 1991). NKR-P1 includes 3 distinct genes of which cDNA have been cloned and named as cDNA 2, 34, and 40. They are located in the region of 14 kb genomic DNA. NKR-P1 gene 2 has 5 introns and 6 exons. Exon 1 encodes for cytoplasmic domain, Exon 2 encodes for transmembrane region

and 3,4 and 5 encode for extracellular region and Carbohydrate recognition domain (CRD). The gene 2, 34, 40 are termed as NKR-P1A, NKR-P1B and NKR-P1C respectively. All five introns begin and end with the characteristic GT-AC dinucleotides. GT-AC rule describes the splicing sites of nuclear gene of many eukaryotes. That is why exons will be splices together by sparing GT-AC of the intron sequences. The NKR-P1 expresses in higher levels in CE, N2B/BTIN and C57 BL mice than in BALB /C and other strains (Giorda et al, 1992). This indicates expression level varies between different strains of mice. Southern blot analysis deduced that NKR-P1 genes NKR-P1 Receptor in peripheral blood of pig do not undergo genomic rearrangement. It is noted that the natural killer complex in mouse chromosome 6 may encode 2 opposing signal i.e. NKR-P1 and ly-49.

NKR-P1 on RNK-16 (rat NK cell line) cells can stimulate phosphoinositide turnover and calcium mobilization. In mouseNK1.1 Antigen has identified by Mab PK136, NKR-P1 expressed in multiple, related forms (Yokoyama, 1993).

The prospective size of PCR product of NKR-P1 in human blood is 573 bp. In pig PCR product is 661bp. In bovine NKR-P1 PCR product is 484bp. In human there is only one NKR-P1A gene sequence available in GenBank. The b34 and b38 product would be cloned for sequencing. The sequences have been determined in 3 different clones.

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