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Different Way of LMP/TAP/MHC Gene Clustering in Vertebrates, Viviparity and Anti-tumor Immunity Failure

Ivan Bubanovic* and Stevo Najman[1]

Department of Obstetrics and Gynecology, Medical Centre-Nis, Serbio and Montenegro; [1]Institute for Biology, University Medical School-Nis, Serbia and Montenegro

Abstract: Class I and class II MHC genes have been identified in most of the jawed vertebrate taxa. In all investigated bony fish species, unlike mammals, the classical class I and class II MHC genes are not linked and even are found on different chromosomes. Linking and clustering of the class I and class II MHC genes is not the only phenomenon clearly detected in the evolution of immune system from cartilaginous to mammals. In all non-mammalian classes the LMP/TAP genes are highly conserved within class I genes region, while these genes are conserved within class II genes region only in mammals. Today we know that LMP/TAP genes in mammals have a crucial role in peptide processing for presentation within class I molecules, as well as in anti-tumor immunity. For these reasons, differences in clustering of LMP/TAP/MHC genes can be responsible for the differences in mechanisms and efficacy of anti-tumor immunity in non-mammalian vertebrates compared to same mechanisms in mammals. Also, the differences in cytokine network and anti-tumor antigens presentation within classes of vertebrates can be explained by the peculiarity of LMP/TAP/MHC gene clustering.

Key words: LMP/TAP/MHC, immunity, vertebrates, mammals, tumor, viviparity

EVOLUTION OF LMP/TAP/MHC MACHINERY

The innate immune system is the only defence mechanism found in invertebrates, but in vertebrates, it is only a part of the immune system. The second, probably the most important part of vertebrate immune system is adoptive immunity. Due to the lack of molecular evidence that vertebrates inherited adoptive immunity from invertebrates, there is the presumption that this part of the immune system was developed as an effective advancement of the innate immunity (Rittig et al., 1996).

Agnatha do not have genes for MHC molecules, although they have the ability to reject transplants. Cartilaginous are evolutionarily the earliest vertebrate group displaying clearly defined MHC class I and class II genes, which indicates that some of their ancestors must have been the precursor of the MHC system. This suggests that class I and class II genes are older than 450 million years (Ohta et al., 2000). Geneticists and molecular biologists still have not identified the gene likely to be the evolutionary precursor of MHC genes. While some authors propose that class II genes are evolutionarily older than class I, others support the idea that class I genes are the earliest molecules of the tissue compatibility. Also, there is a real presumption that the precursors of class I molecules are actually heat shock proteins (HSP) (Lawlor et al., 1990; Flajnik et al., 1991; Hughes et al., 1993). The absence of MHC genes in jawless fish or invertebrates suggests that the MHC arose rather abruptly in a jawed vertebrate ancestor, probably a *placoderm* (Kasahara et al., 1992; Okamura et al., 1997). One hypothesis suggests that genome-wide duplications played a role in the emergence of the MHC and the entire adaptive immune system (Kasahara et al., 1996), as genes linked to class I and class II are found in four paralogous clusters in mammalian genomes (Boyson et al., 1996; Kasahara et al., 1997). In all tetrapod species examined to date including several primates, class I and class II genes are closely linked in the bird *Gallus* (Gyllensten et al., 1989) and the amphibian *Xenopus* (Moriuchi et al., 1985; Kasahara et al., 1992). However, among older taxa, in all investigated bony fish species, including the zebrafish (Karr et al., 1986), carp, salmon (Nei et al., 1997), and trout (Parham et al., 1996), classical class I and class II genes are not linked and even are found on different chromosomes. It

*To whom correspondence should be addressed.
E-mail: ibubanovic@yahoo.com

was proposed that one of two scenarios occurred in vertebrate evolution (Karr et al., 1986; Parham et al., 1997): (i) class I and class II genes arose on different paralogous chromosomes in a jawed vertebrate ancestor and "clustered" together in a tetrapod ancestor, or (ii) the genes were originally in the same linkage group but were rent apart in a recent teleost ancestor and now lie on different chromosomes in this single vertebrate lineage (Hughes et al., 1988).

Regarding the tendency towards the linking and clustering of class I and class II genes along the evolution of vertebrates, it seems logical to ask: what is the nature of the selection pressure that directed the development of this phenomenon? Almost every argument on the evolution of the immune system puts microbes into the foreground as the source of the strongest evolutionary pressure that modelled the vertebrate immune system. However, could microbes really be considered the only factor of evolutionary pressure that could have led to the clustering of class I and class II in higher vertebrates? Linking and clustering of the class I and class II genes is not the only phenomenon clearly detected in the evolution of the immune system from cartilagofish to mammals. In birds (Kaufman et al., 1995), bonyfishes (Karr et al., 1986) and reptiles, but not in mammals, the genes responsible for determination of LMP and TAP molecules are linked with the class I genes. This phenomenon is most striking in zebrafish, because the class I gene complex, LMP and TAP are found on the same chromosome, while class II genes are found on a quite different one. It is presumed that MHC III genes were inserted into the MHC in the later evolution of vertebrates, but some data suggesting that the genes which determine the C4 component of complement and HSP70 in reptiles and mammals are linked with the class I, also propose that this phenomenon occurred in the common ancestors of reptiles and mammals (Kasahara et al., 1992, 1996, 1997). This assumption is in corollary with the hypothesis that the class I and II genes appear to have the common precursor gene. Meanwhile, these data also indicate the likely scenario of the MHC gene evolution, where MHC class I and class II had quite independent evolutionary pathways, whereas classes I and III probably had the common evolution.

From the gene arrangement on the chromosome map of different vertebrates, it is clearly noticeable that in all non-mammalian classes the TAP and LMP genes are highly conserved within class I genes region, which suggests that the processing and expression of genes take place on the same cluster. In mammals, TAP and LMP genes are highly conserved evolutionarily within class II genes region (Kasahara et al., 1996).

Available evidence suggests that the genetic content and level of MHC complexity are comparable in all mammalian species. However, several intriguing peculiarities have been identified in equine and cattle. Recent genetic mapping suggests that MHC of horses may be disrupted even more than it is in chickens. This would be the first example in mammals where MHC sequences are located on different chromosomes (Fraser et al., 1998). Surprisingly, horse homologue to TAP2 is conserved in MHC class II region. Also, TAP2 genes and class II surrounding genes in horses order seems to be fairly well conserved with the human class II organization (Personal communication with Dr. Antczack, J.A. Baker Institute for Animal Health College of Veterinary Medicine Cornell University Ithaca, NY 14853).

VARIABILITY OF TAP/LMP/MHC GENES

In mammalian class II region of MHC, four genes have been described to be implicated in processing of MHC class I presented peptides. Two of these are TAP1 and TAP2 encoding for ER membrane transporter proteins and the other two are LMP2 and LMP7 for proteasome subunits. These genes are polymorphic, although much less so than classical MHC class I and II genes. There is a controversy concerning the possible functional implications of this variation.

The molecular and functional analyses of rat and primate TAP2 homologues indicated major differences in gene diversification patterns and selectivity of peptides transported. The sequence analysis of the TAP2 cDNAs from gorilla EBV virus-transformed B-cell lines revealed four alleles with a genetic distance of less than 1%. The diversification of the locus appears to have resulted from point substitutions and recombinational events. Evolutionary rate estimates for the TAP2 gene in gorilla and human closely approximate those observed for other hominoid genes. The amino acid polymorphisms within the gorilla molecules are distinct from those in the human homologues. The absence of ancestral polymorphisms suggests that gorilla and human TAP2 genes have not evolved in a trans-species fashion but rather have diversified since the divergence of the lineages (Loflin et al., 1996). Polymorphism within these genes could alter the level of the immune response, a phenomenon relevant to the development of auto-immune diseases. For example, Moins-Teisserenc et al. (1995) investigate that TAP2 gene polymorphism contributes to the genetic susceptibility to multiple sclerosis. Similarly with previous citation, Martinez-Laso et al. (1994) found that TAP2 genes are placed within the HLA complex, have limited genetic variability and encode two main groups of TAP, the so-called TAP2×01 alleles, with a short ATP-binding domain, and the TAP2×0201 allele with a long domain. The shorter TAP2×01 alleles are present in 99% of diabetics and 90% of controls.

TAP and LMP genes are undoubtedly polymorphic, but if microbes maintain MHC diversity, then why are other genes that influence disease resistance not as polymorphic

as the MHC? The MHC is widely cited as an example of genetic diversity driven mainly by viruses, yet the largest survey on MHC and disease resistance found evidence for directional selection (Hill et al., 1991), which reduces genetic diversity. There are several possible reasons for this inconsistency (Bubanovic et al., 2004a, 2004b):

1. Disease resistance genes are generally polymorphic, but the variation is hidden and will require molecular techniques to uncover. This explanation seems unlikely because most major immune system genes, such as TCR and Ig genes, are not particularly polymorphic;
2. MHC genes are unusually polymorphic because their role in the immune reaction is qualitatively different from other genes such as TCR, Ig, TAP, LMP and RAG. The absence of high TAP/LMP gene variability can be explained by such a stable intracellular antigen processing machinery;
3. In mammals, the TAP/LMP genes are located within the class II region but they control antigen presentation associated with class I molecules. Therefore, microbe evasion should provide a similar selective force on TAP/LMP and MHC genes. Accordingly, it is likely that the evolutionary pressure of microbes as well as the other selection pressures may also have acted to favour high MHC variability and relative evolutionary conservation of TCR, Ig, TAP, LMP, and the RAG genes.

Although both MHC molecules and TCR are known for their extreme degrees of diversity, the underlying mechanisms are fundamentally different. Whereas TCR owe their diversity to special somatic diversification processes, MHC molecules have mutation rates similar to those of most other genes (Parham et al., 1995). An explanation for the high degree of MHC polymorphism cannot be sought in vertebrate allograft rejections, as these are experimental artefacts and thus not naturally involved in evolutionary selection, probably until the emergence of viviparity (De Boer et al., 1995; Bubanovic et al., 2004a, 2004b). One of possibility is that the vertebrate MHC polymorphism is a "relict" of ancestor genes polymorphism (Buss et al., 1985). Alternatively, the selection pressure for MHC diversity may be due to peptide presentation to the immune system. Several most commonly held views are that MHC polymorphism is due to selection favouring MHC heterozygosity and evolutionary accumulation of MHC molecule diversity or due to the selection for hosts with rare MHC molecules (Bodmer, 1972; Doherty et al., 1975).

Regarding the role of MHC molecules in the anti-microbe defence and antigen-presentation to the immune system, the number of MHC genes expressed per individual is surprisingly small. For example, each human individual expresses maximally six different classical MHC class I genes, and twelve different MHC class II molecules. One would expect evolution to favour the expression of many MHC genes per individual. A solution to this paradox has been sought in "self-non-self" discrimination. A widely accepted argument is that an excessive expression of MHC molecules leads to the depletion of the T cell repertoire during "self" tolerance induction. In addition, the highly polymorphic MHC genes control immunological "self-non-self" recognition; therefore, the polymorphism may function to provide "good genes" for an individual's offspring. There are three adaptive hypotheses for MHC dependent survival under evolutionary pressure of microbes (especially viruses): (i) the consequence of high MHC genes variability is mainly MHC-heterozygous offspring that may upgrade anti-microbe immune response. Although this hypothesis is not supported by tests of single microbe infection, MHC heterozygotes may be resistant to multiple viruses or other microbes; (ii) MHC variability enables hosts to provide a "moving target" against rapidly evolving microbes that escape immune recognition. Such viruses are suspected to drive MHC diversity through rare allele advantage. Thus, the two forms of viruses-mediated selection thought to drive MHC diversity, heterozygote and rare allele advantage, will also favour MHC variability and (iii) the diversification of MHC genes may also function to avoid inbreeding; a hypothesis consistent with other evidence that MHC genes play a possible role in kin recognition or even bifurcation of species from subspecies and emergence of a new species.

The MHC loci are known to be highly polymorphic in humans, mice and certain other mammals, with heterozygosity as high as 80-90%. Six different hypotheses have been considered to explain this high degree of polymorphism (Bubanovic et al., 2004a, 2004b):

1. A high mutation rate with gradual accumulation of spontaneous mutational substitution over evolutionary time. The main source of the variability in the MHC genes sequences is a point mutation but the mutation rate is by no means higher in the MHC than elsewhere in the genome. Because of transspecies polymorphism, the accumulation of point mutations over evolutioanry times (millions of years) results in the extensive polymorphism;
2. Gene conversion, interlocus genetic exchange or periodic intragenic (interallelic) and more rarely, intergenic, recombination within the class I genes;
3. The selection against mutational divergence in the regions of the class I molecule involved in T cell receptor interaction and also in certain regions that interact with common features of antigens;
4. Positive selection pressure in favour of the persistence of MHC polymorphism and heterozygosity at the antigen recognition site;
5. The negative selection of the MHC alleles associated with tolerance to microbes;

6. Microbes-exerted the negative selection of low polymorphic or monomorphic MHC genes.

EXPRESSION OF LMP/TAP/MHC MOLECULES BY TUMOR AND TROPHOBLAST CELLS

In many species of eutherian mammals, the mechanisms of pregnancy survival due to the reducing of placental expression of the MHC genes. Unexpectedly, in some species the MHC expression is often re-established in the most invasive trophoblast cells. It is not known why the transplantation antigen expression in the fetal cells most exposed to the maternal immune system is advantageous. It is possible that such an expression aids the process of invasion or exerts an immuno-protective effect on the fetus. It may prove possible to identify the essential steps that all eutherian fetuses take to ensure their survival in the face of the potential maternal immune attack by studying the common features of the placental immunology of different species (Bubanovic, 2004b).

There is a large body of data that in most mammals, the fetus limits its presentation of the paternal MHC molecules to the mother immune system. In the horse, however, functional, polymorphic MHC class I antigens are expressed at high levels on the invasive trophoblast cells of the chorionic girdle between days 32 and 36 of pregnancy, although not on the adjacent non-invasive trophoblast of the chorion and allantochorion membranes. Bacon et al. (2002) found that 33-34 days old conceptus tissue revealed both transcriptional and posttranscriptional regulation of cell surface class I expression in horse trophoblast. The invasive class I positive trophoblast showed levels of steady-state mRNA nearly as high as those in lymphoid tissues from adult horses, whereas non-invasive class I negative trophoblast also contained transcripts for class I, but at lower levels similar to those present in adult horse non-lymphoid tissue. Also, the source of fetal MHC antigens in the pregnant mare appears to be the specialized trophoblast cells of the chorionic girdle region of the developing placenta. These cells invade the endometrium between days 36 and 38 after the ovulation to form the endometrial cups. The progenitor girdle cells express the high levels of paternal MHC antigens, while the non-invasive trophoblast cells of the allantochorion and the differentiated trophoblast cells in the mature endometrial cups do not. This expression of MHC antigens by the chorionic girdle cells is unusual for a trophoblast tissue, and differs from most forms of trophoblast studied in other species (Antzak et al., 1989).

In pig, trophoblast becomes attach to the endometrial epithelium of the uterus between days 14 and 22. Around this time, the outer endodermal surface of the developing allantois begins to fuse with the inner endodermal layer of the chorion, starting at the embryo and progressing to the both ends of the elongated blastocysts. The mesoderm then develops between the two endodermal layers to innervate the allanto-chorionic sac. Practically the entire surface of the allanto-chorion forms the placenta, hence the name placenta diffusa. The trophoblast remains a non-invasive single layer in the pig. Beginning around midgestation, the capillary plexuses at the tips of the chorionic villi penetrate between the trophoblast cells to about 2 μm from maternal epithelium at term (Ramsoondar et al., 1999).

The lack of the polymorphic MHC molecules on pig trophoblast follows that of both the sheep (Gogolin-Ewens et al., 1989) and the horse, in which, except for the expression of class I MHC on the invasive trophoblast of the transient chorionic girdle cells, the non-invasive trophoblasts of the chorioallantoic membranes are class I negative (Donaldson et al., 1990; Maher et al., 1996). In contrast, in the cow placenta, which is structurally similar to that of the sheep (syndesmochorial), the non-invasive trophoblast of the interplacentomal allanto-chorion has been found to express class I in some instances (Low et al., 1990). This is especially perplexing since the same monoclonal anti-sheep class I antibody (SBU-1) was used in both the cow and sheep studies (Gogolin-Ewens et al., 1989; Low et al., 1990). Since the monoclonal antibodys (mAb) is directed against pig thymocytes, cells that very likely do not express monomorphic, pregnancy-associated class I MHC antigens, it may not recognize these unique forms; hence, the possibility that monomorphic forms are expressed on pig trophoblast cannot be formally excluded. Human extravillous trophoblast subpopulations express the HLA-G molecules (Mc Master et al., 1995), while the basal trophoblast of the rat expresses the so-called the PA molecules (Macpherson et al., 1986) and both are unique monomorphic class Ib molecules. Recently, HLA-G was shown to be restricted to a differentiated cytotrophoblast (McMaster et al., 1995), to be co-dominantly expressed in first-trimester trophoblast cells (Hviid et al., 1998), and to present peptides in a manner similar to that of polymorphic class I HLA molecules (Diehl et al., 1996). The mAb directed against a monomorphic determinant of class I MHC detects both polymorphic and monomorphic MHC antigens in humans (Barnstable et al., 1978). Therefore, it is possible that the mAb would also detect putative monomorphic forms of class I MHC molecules in the pig.

In the developing human embryo, trophoblasts directly contact the maternal tissues and could be the targets for the maternal immune cells. Extravillous interstitial and endovascular trophoblasts that invade the uterus and uterine blood vessels in early pregnancy also express the non-classical MHC class I molecules HLA-G and classical HLA-C. The mRNA for another non-classical MHC class I molecule, HLA-E, also is expressed on the placental tissues. The recent demonstration with *in vitro* experiments that HLA-G can activate the KIR expressed on the cells of

lymphoid and myelomonocytic origin is consistent with the hypothesis that HLA-G plays an important role in establishing the maternal-fetal tolerance (Hviid et al., 1998).

There are two major pathways of antigen processing within the APCs and target cell: the endogenous and exogenous. The endogenous pathway processes proteins that have been synthesised within the APCs. The tumor associated antigens undergo the same or similar processing, as all other intracellular and extracellular molecules which are presented to the effectory immune cells through APCs. The processing of antigens is a multi-step process which involves: antigen uptake, degradation of the molecules, binding of fragments (peptide) to the newly synthesized MHC molecules, transport and expression of the MHC molecule/peptide complex on the cell surface (Germain, 1994; Cella et al., 1996, 1997).

As with all cytoplasmic proteins, the "non-self" molecules are continuously degraded via the 26S proteasome complex into 8-10 amino acid long fragments. Proteasomal components also include molecules such as LMP2 and LMP7. Proteasome complex activity varies from minimal to very high. One of the most important activators of proteasome, related to the immune reaction and final presentation of antigens, is IFN-γ. In mammals, LMP2 and LMP7 genes are associated with class II genes on the same chromosomal sequence, which makes the mechanism of antigen processing and class II molecules expression a joined, well-coordinated action (Cella et al., 1997; Lobigs et al., 1999; Zhang et al., 2001).

The expression of cell surface MHC class I/peptide complex requires a coordinated transcription of multiple genes such as MHC class I heavy chain, β2m, TAP1, TAP2, LMP2 and LMP7. All of these genes are expressed and defined at distinct levels in normal tissues, and are inducible by IFN-γ. There are two independent elements that are sufficient to activate the transcription of a reporter gene. One (hereby called TAP2 P1) is located 5' to the TAP2 exon 1, while the other (hereby called TAP2 P2) is a transcription initiator residing in intron 1. The analysis of the 5' sequence of TAP2 mRNA indicates that both promoters are active. Moreover, while the TAP2 promoter region contains *cis* elements that can mediate TAP2 induction by IFN-γ, such as γ-activation site and IFN response factor binding element (IRFE). Only the IRFE is required for IFN-γ induction of TAP2 promoter *in vitro*. The IRFE appears to work as an enhancer for the initiator (P2). Together with another promoter recently identified by others, TAP2 therefore has three independent promoters that can be differentially regulated (Lobigs et al., 1999; Knittler et al., 1999).

During proteasome mediated degradation of antigens, the MHC molecules are synthesized in ER, but before the products of proteasomal activity bind the MHC molecule, they first need to be transported from cytosol to ER. TAP1 and TAP2 are known to mediate in the control of immune reaction, and function as the transporters of peptides. The importance of their function has been demonstrated on the cells with mutated TAP genes (Lobigs et al., 1999; Knittler et al., 1999). This mutation results in the lessening of the number of expressed MHC molecules, i.e., an aberrant intracellular expression of the MHC/peptide complex. The transfection of the normal TAP genes into mutant cells restores the stable surface MHC expression. The MHC molecules which did not bind the peptide cannot be expressed on the cell surface. Moreover, they show a high instability, so they are sent to undergo a proteolytic degradation and recycling. TAP activity can be changed under the influence of various factors such as: cytokines, viruses, hormones, prostaglandine etc. The dependence of the MHC expression on the activity of TAP molecules can be demonstrated on the cell infected with the herpes simplex virus (HSV) and adenoviruses.

HSV inhibits TAP1 and TAP2 proteins, preventing the processing of the peptides towards MHC molecules. Most of the MHC molecules which have not bound the peptide are being recycled, while only a small number of those binding the viral or some other peptide will be expressed on the cell surface. The HSV-infected cell expresses a low number of the MHC molecules, and will most probably be eliminated by NK cells, not by CTL. Adenoviruses "block" the already expressed MHC/peptide complex, thus inhibiting its recycling and increasing its half-time. The result of adenovirus activity is the increase of the number of MHC/peptide complexes (mostly non-viral) on the cell surface (Lobigs et al., 1999; Knittler et al., 1999; Zhang et al., 2001).

It is necessary for the activation of effectory mechanisms of anti-tumor response that the antigen, i.e. its peptide, is first presented to the T cells on APCs. Although macrophages and partly B lymphocytes play the role of APCs, dendritic cells have shown the highest potential for stimulating the anti-tumor immune response (Cella et al., 1996, 1997). Non-activated, or commonly defined as "immature", dendritic cells (DCs), whether they are Langerhans or the so-called intestinal DCs, have a high potential for incorporating the antigens from the external environment. The mechanism of antigen internalisation by DCs works mainly in two ways. According to the first model, antigen internalisation is carried out via mechanism of macropinocytosis, while the second model precludes the interaction of membranous receptors (mannose or Fc-receptor). From the aspect of stimulation of anti-tumor effectory mechanisms, it is important whether the antigen will be internalised via pinocytosis or the mannose-receptor. The effectory mechanisms developing after "sugar-dependent way" of antigen internalisation are 100-10,000 times more intensive than those developing after the internalisation of antigens

via pinocytosis. The mechanism of antigen molecule processing after the internalisation via Fc-receptor depends on the proteolytic activity of proteasome, whereas the expression of MHC/peptide complex depends on the transporting mechanisms including TAP1 and TAP2 molecules (Cella et al., 1996, 1997).

CONCLUSION

The role of LMP/TAP/MHC machinery is very important for "quality" and efficacy of the immune response. In addition, this fundamental control mechanism of immune reaction is responsible for various forms of immune tolerance of proliferative tissues, such as trophoblast and tumors. Comparative genomic researches show that linking and clustering of LMP/TAP/MHC genes is not the same in different classes of vertebrates, so that the control of the immune reaction associated with these genes probably differs from class to class of vertebrates. The phenomenon of linking of LMP/TAP/MHC genes in mammals and "escaping" the TAP genes from MHC class I region in this vertebrate group might be associated with immune related compromise of viviparity and, consequently, may be a source of anti-tumor immunity failure.

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