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Review Article

Precision Medicine and the Use of Cryptic Peptides as a Source for MHC I Epitopes

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Abstract

The recognition of epitopes restricted to the major histocompatibility complex (MHC) by the cellular immune system plays an important role in identifying neoplastic change or infectious disease. Accordingly, it is crucial to understand the genetic bases of epitope production. Herein, we review non-conventional methods of producing MHC I epitopes from translation of genetic information derived from non-coding regions thought to be nonfunctional. The investigation of these so-called cryptic epitopes has paved the way for the development of new management tools to treat challenging medical problems. Specifically, the production of immunotherapies for cancer and viral infections have now emerged as a realistic prospect. Given the variability of genetic information within cancers, targeting cryptic epitopes comes with dilemma of needing to tailor a given therapy to the individual patient. This precision medicine approach raises both scientific questions and logistic problems that need to be streamlined to gain satisfactory results.

Keywords: Major Histocompatibility Class I; Cytotoxic T Lymphocytes; Immunopeptidome

Introduction

Cell surface major histocompatibility class I (MHC I) molecules provide an important tool for immune surveillance. They act as mirror reflecting the inner state of the cell by binding antigen MHC I associated peptides (MAP) of a variable 8 - 11 amino acids in length that are collectively referred to as the immunopeptidome [1,2]. The $CD8^+$ Cytotoxic T Lymphocytes (CTL) receptors then recognize these MAP and either kill or decrease replication of the MAP expressing cells. This CTL surveillance for MAP has an important role in both eliminating virally infected and tumor cells but can cause autoimmune diseases and transplant rejection as well [3]. That is why it is important to appreciate the source and characteristics of MAP, as they are the key to understanding the immune response.

The immunopeptidome samples 2% of the cell proteome made up from approximately 10,000 proteins that are about 500 amino acids long [2,4,5]. It was originally thought that the protein source for the MAP was derived solely through the conventional translation pathway. Translation begins with the eukaryotic initiation factor 2 (eIF-2) binding with Met-$tRNAi^{Met}$ and engaging the smaller 40S ribosome subunit to form the 43S initiation complex. The mRNA is then scanned from the 5' capped end until it reaches the AUG start codon at an open reading frame (ORF). At that juncture, eIF-2 detaches from the complex with the help of eIF-5 and leaves the Met-$tRNAi^{Met}$ in place bound to the AUG by its anticodon. The larger 60S ribosome subunit then binds to the 40S-Met-$tRNAi^{Met}$ complex to form the 80S ribosome that translates the mRNA with the help of other elongation factors [6-8]. Eventually, the ribosome reaches a stop codon and detaches from the mRNA with the help of release factors to leave the formed protein [2,9].

To form a MAP, the intracellular protein has first to be enzymatically digested by the proteasomes in the cytoplasm forming MAP precursor [10] that is transported to the endoplasmic reticulum by the transporter associated with antigen (TAP) complex [11]. Following trimming of the N-terminal with amino-peptidase to reach the optimal amino acid length [12], tapasin loads the peptide onto the MHC I forming peptide MHC I complex (pMHC I) to be expressed on the cell surface [13]. This process may be inhibited by several herpesviruses [14].

Cryptic peptides - old theories and novel discoveries

The idea that the conventional translation pathway was the only source for producing MAPs was questioned thirty years ago when cryptic peptides were characterized and found to be produced by non-conventional synthesis. In 1989, Boon et al cloned and expressed P91A into a mouse tumor cell line that carried a point mutation in exon 4a of the mice gene P91 [15] that rendered the cell susceptible to CTL killing. Surprisingly, the same response was observed with transfection of exon 4 and surrounding introns without an expression vector. They then proposed the pepton hypothesis that short subgenic regions can form pepton-RNA that yield antigens that don't require further digestion before peptide loading onto the MHC I [16].

In 1996, Yewdell., *et al*. suggested additional mechanisms for the unusual sources of MAP and argued that peptidomes could be produced as defective ribosomal products (DRiPs) by the premature termination of protein production, or abnormal folding of proteins. These abnormally biosynthesized products were then rapidly hydrolyzed by proteasomes forming the MAP [17]. Indeed, the rapid advancement of proteogenomic science has revealed that sub genomic regions of DNA thought to be transcriptionally inactive may actually lead to protein formation. We now know that 75% of the genome can produce proteins expanding the list of the described cryptic peptides [18].

Where to initiate?

Several non-conventional mechanisms of translation have now been described. For example, the initiation of translation through internal ribosomal entry site (IRES) is central for the translation of non-caped mRNA containing multiple start codons [19,20]. For example, the HLA-A*0201 restricted melanoma antigens are derived from a polycistronic mRNA containing IRES on multiple short ORF from the *meloe* gene. These encode the MELOE-1 and MELOE-2 antigens, which trigger CTL responses to melanoma cells but not healthy melanocytes [21-23]. To demonstrate that the neoantigen presentation in the melanoma cells was IRES dependent, the phenotype was recapitulated by introduction of a DNA fragment of the region between ORF MELOE-2 and ORF MELOE-1 containing the IRES sequence [23].

Other mechanisms have been shown to producing cryptic epitopes on the level of translation initiation. These include initiation codon scan through by ribosomal avoidance of translation at the first AUG to initiate at the second [24]. A similar mechanism has been described where the ribosome does not dissociate after completing translation at a stop site and reinitiates translation at the following AUG [25]. These mechanisms may over ride point mutations inducing premature termination at stop codons permitting the ribosome to produce proteins in a non-conventional reading frame [26].

In addition, the ribosome also has the ability to start at near-cognate codons that differ from AUG by one nucleotide, such as ACG and GUG. These sites have been characterized by causing ribosomes to accumulate at sites of translation and then sequencing ribosome protected RNA; a process referred to as ribosome profiling [27]. These near-cognate codons can be translated to Methionine but in a level that is 10 - 30 fold less efficient than AUG [28]. The exact mechanism is still debated as initiating translation either upper stream, downstream, or overlapping the protein-coding regions [29,30]. CUG appears to be the most efficient in starting translation and the codon is translated into Leucine in a fashion distinct from the conventional initiation method [27,31,32]. Leu-tRNA can form initiation ribosomal complexes within the eIF2A pathway instead of the classic eIF2, as demonstrated by knocking down the eIF2A [31]. In follow up studies, Horng., *et al*. showed that CUG is not being translated as Leucine and then continuing translation through a leaky stop codon by a series of experiments using vectors with multiple stop codons that could be translated in the three reading frames [33]. Taken together, these studies show that non-conventional methods of initiation seem to be an important tool for producing non-cryptic peptides and translation can also occur in non-conventional ways.

Slippery Elongation

Another non-conventional mechanism of producing MAP can occur during elongation, where the tRNA can slip one or two nucleotides to change the reading frame. As a result, the codons sequences change during translation to produce a hybrid protein from two separate frames. This mechanism is important for retroviruses with limited space in their genome [34] permitting production of more proteins from limited genetic information [2]. This mechanism has been demonstrated in human cells as well [35].

Two types of frame shifting have been reported. Programmed ribosomal shifting (PRF) is the most common, where the ribosome pauses on a slippery site of seven-nucleotide to permit the tRNA to slip and change the reading frame. The slippery site is followed

by a spacer of fewer than 12 nucleotides and a pseudoknot. This structure regulates the PRF process by blocking the action of the helicase protein, halting the elongation factors and therefore, allowing the tRNA to shift the reading frame [36,37]. A well characterized example of PRF has been shown with HIV where a +2 frame shift from a region that overlaps the *gag* gene of HIV-1 produces a cryptic peptide referred to as Q9VF. This peptide is restricted to HLA-B7 and immunization of transgenic mice expressing human HLA-B*0702 triggers a CTL proinflammatory response with IFNγ production [38,39].

Incidental frame shifting has a lower frequency of inducing frame shift as compared to PRF [40]. Herpes simplex virus has *thymidine kinase* gene that may escape acyclovir due to a slippery seven-guanine nucleotide sequence; this activity produces MAP that can trigger a CTL response [41,42].

Leaky brake system

Ribosomal failure at stop codons that serve to terminate translation can also serve as a tool for producing cryptic MAPs within the 3'UTR. For example, specific aminoglycosides can induce a ribosomal read through by binding the ribosome and preventing the t-RNA anticodon from recognizing the leaky termination codons. Accordingly, the ribosome continues translating without interruption. This discovery is now studied as a therapeutic approach for genetic diseases that prevent the formation of complete functional proteins, because of premature stop codons (PTC), such as Duchene Muscular Dystrophy. Accordingly, these aminoglycosides are capable of producing a complete functional protein PTC, to some degree [43].

As a result of stop codon read through, MAP can be generated through the expression of 3'UTR to produce neoantigens that may trigger autoimmune reactions. To show that aminoglycosides can induce production of cryptic MAP, Goodenough and colleagues cloned different constructs within the vaccinia virus genome that could express the SIINFEKL peptide downstream of influenza nucleoprotein (NP) sequence with various stop codons in between. After infecting mice pretreated with gentamicin, they harvested the mice splenocytes, and tested the CTL response against the SIINFEKL peptide by ELISpot. They found that the CTL from gentamicin treated mice produced higher amounts of IFNγ than untreated mice demonstrating the ability of aminoglycosides to produce peptides through stop codon read-through [44].

Transcriptional and post-translational mechanisms

Non-conventional mechanisms for cryptic MAPs production are not restricted to the translational level only and all the levels of protein production may result in neoantigens. For example, antisense transcription of the *RU2* gene results in the production of cryptic MAP recognized by CTL in Renal Cell Carcinoma patients [45]. Post-translational modifications may also result in MAP. For example, the protein product of the tyrosinase gene in melanoma cells where asparagine is converted to aspartic acid post translation to forms a peptide that triggers a CTL response [46]. Accordingly, MAP may be created by different transcriptional and the post-translation events.

Precision Medicine and the use of Cryptic peptides

Uncovering the various sources of cryptic peptide production offers novel approaches to treat challenging disorders resistant to current therapy. In the oncology field, for example, conventional peptides used to boost immune responses to tumor antigens have limited effect due to tolerance induction within the tumor [47]. The use of MELOE-1 and 2 peptides expressed exclusively in melanoma cells elicits immune CD8+ T cell response suggesting that these antigens may be good candidates for an anti-tumor vaccine. More recently, RNAseq has been used to describe multiple cryptic peptides in melanoma patients with metastatic disease to vastly expand the list of the tumor specific antigens that can be used for tumor vaccines. In this example, next generation sequencing of the tumor transcriptome and genomic DNA provides data on mutations occurring within the tumor as well as the relative expression of the presumed neoantigen. Further bioinformatic processing reveals potential neoantigens that may be expressed by the patients HLA to provoke anti-tumor CTL. Then peptide vaccines can be synthesized for testing against the patients PBMC or tumor infiltrating lymphocytes, if available, to determine the potential efficacy. This precision medicine approach is costly but effective. However, the use of tumor generated MAP as neoantigens may also be limited by tolerance induction within tumors. Nevertheless, the use with check-point inhibitors in combination with multiple novel cryptic peptides is now providing long lasting anti-tumor effects [48,49]. An example for failure of targeting cryptic peptides due to tolerance is the vaccination with a vascular endothelial growth factor derived cryptic peptide in patients with renal cell carcinoma that failed to promote an active CTL response. The peptide was found expressed in normal tissues to a lesser degree than observed

within renal cell carcinoma tissue, but to an extent that it induced tolerance [50].

Investigators have turned their attention to treating viral disorders as well using immunotherapy against cryptic peptides. For example, Ho and colleagues have carried out adoptive transfer experiments targeting virally infected cells. After infecting CD 8^+ T cell KO mice with murine AIDS, they adoptively transferred CTL specific for a cryptic peptide derived from +1 ARF of the *gag* gene of LP-BM5 three days later with IL-2 to stimulate lymphocyte replication [51]. The infected KO mice that received adoptive transfer had close to undetectable viral load as compared to control KO mice that didn't receive adoptive transfer [52]. While targeting viral cryptic peptides for infection treatment in humans haven't shown reliable advancements, similar experiments are now being performed in transplant recipients to control Epstein Barr virus infection targeting conventional peptides [53,54]. However, these precision medicine studies are logistically challenging and require much fine tuning before they can be used in clinical practice. Several questions remain unanswered concerning their efficacy and the ability to measure immunologic activity, using a Quantiferon™ assay to measure response, for example [55].

Targeting of cryptic peptide production is also a consideration for future research as these neoantigens have been implicated in the pathogenesis of different diseases. CTL directed against cryptic peptide driven by IL-10 gene has been identified in the synovial fluid of a patient with Reiter's syndrome [35]. A cryptic peptide driven from the 5' UTR region of *TMSB4Y* gene on the Y chromosome has been found to promote chronic graft versus host disease in a male patient receiving hemopoietic stem cell transplant from his HLA identical sister [56]. Accordingly, cryptic peptides are implicated in different disorders mandating novel approaches for immunotherapy.

Conclusion

In summary, our understanding of cryptic peptides has improved over the last three decades to an extent that novel therapies using precision medicine approaches are moving into translational research in the clinic. However, there is still much to learn before they enter clinical practice.

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