Contribution of Inducible Nitric Oxide Synthase to the Transformation of HTLV-1 Infected CD4+ T-Cells

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Abstract

The Human T-cell Leukemia Virus type 1 (HTLV-1), is the first retrovirus associated with a human cancer. HTLV-1 is the causative agent of an aggressive and fatal malignancy of CD4+ T lymphocytes known as Adult T-cell Leukemia lymphoma (ATLL). Since the discovery of the virus in 1980, intensive investigations have been undertaken to determine how HTLV-1 drives the transformation process in infected cells. This is because the oncogenic features of HTLV-1 make it an excellent tool to dissect the molecular pathways involved in cancer development. More important, HTLV-1 induced leukemia is a typical inflammation-mediated malignancy with constitutive activation of the NF-kB pathway, which is also a critical determinant in many other cancers. How NF-kB contributes to the leukemogenic process is not completely defined. We recently demonstrated that the NF-kB pathway induces the expression of inducible nitric oxide synthase (iNOS) in HTLV-1 induced leukemia. iNOS enzymatically generates nitric oxide, which is an oxidative and nitrosative agent of DNA and proteins. Nitric oxide was found to be associated with a large number of DNA Double Strand Breaks (DSBs) in HTLV-1 transformed cells. Here, we will review the major effects of nitric oxide on HTLV-1 induced leukemia.

Keywords: HTLV; Nitric oxide; Tax

Introduction

The Human T-cell Leukemia Virus type 1 (HTLV-1) is the etiological agent of Adult T-cell Leukemia Lymphoma (ATLL), a rare and aggressive T-cell malignancy. The transmission of the virus occurs sexually or by IV drug abuse, but the most efficient way of viral transmission is through breast-feeding from an infected mother to her baby [1,2] (Figure 1). This is because the breast epithelial cells regulate a physiological recruitment of lymphoid and myeloid cells from the circulation into the milk, while secreting nutritive molecules, antibiotic substances, growth factors, inflammatory cytokines, and chemokines [3]. As a result, breast milk allows contact between lymphoid cells which promotes cell to cell transmission of the virus, a more efficient manner of virus spread as compared to free particle infection [4,5]. Yet, for unknown reasons, only a few percent of infected individuals develop ATLL after a long period of latency [6]. Currently, there is no way to predict which infected patients will develop ATLL, and there is no effective treatment for those entering the acute phase of the disease. Of note, it is still not known whether the integration of the proviral DNA into specific loci in the human genome has a role in ATLL development [7]. Moreover, the concept of the monoclonal disease development has recently been debated as a result of deep sequencing results, which showed that multiple clones can evolve during progression of the disease [8]. It is also not understood why ATLL develops only in CD4+ T-cells, while the virus is present in almost all lymphoid and myeloid progenitors, including hematopoietic stem cells (HSC) [9,10]. Data obtained from HTLV-1 infected humanized mice (HIS) demonstrated that high a frequency of HTLV-1 infection was found in the double positive T-cells during lymphogenesis suggesting that lymphoid progenitors constitute the niche of HTLV-1 infection. The other infected cells either represent the latent reservoirs of the virus or lack properties to support the process of transformation [11-14]. Because HTLV-1 infection has evolved mechanisms that activate CD4+ T-cells and impair the immune CTL response, the outcome of the disease largely depends on two antagonist factors, the proviral load and the efficiency of the immune response against the infected cells [6,15]. Activation of proliferation and inhibition of tumor suppressors are also two major hallmarks of oncogenic events occurring during the long period of latent infection. However, the accumulation of genetic defects is believed to be a driving force for transformation [16]. How and when these genetic defects accumulate is still under intense investigation.

Over the last decade, there is increasing evidence that inflammation is a hidden force that drives many malignancies [17-20]. More than 90% of cancers are associated with some forms of chronic inflammation, which are induced by infections, obesity, use of tobacco, and exposure to different mutagenic agents. Most likely, inflammation is related to cancer through processes that involve genotoxicity, aberrant tissue repair, proliferative responses, invasion and metastasis. In contrast to somatic inherited mutations, inflammation may induce random mutations, which will contribute to additional cooperating events for the initiation and maintenance of the transformation process [21,22]. HTLV-1 induced leukemia is a typical inflammation-mediated malignancy with constitutive activation of the NF-kB pathway [23], which is also a critical determinant in many other cancers [24-29]. The NF-kB pathway activates the expression of a large number of genes involved in immunity and the inflammatory response, apoptosis, proliferation, differentiation, and survival [30,31]. Cytokines and chemokines are the first effectors of the inflammatory response [32]. They contribute to the proliferation of pre-neoplastic
cells, but little is known about the downstream effectors that are involved in this process, and whether a specific connection exists between NF-kB activation and accumulation of genetic defects [25].

Figure 1: Characteristics of HTLV-1 associated malignancy. Number of infected individuals and their world endemic distribution as well as the mode of the virus transmission and the prevalence of the disease. Although HTLV-1 infect other hematopoietic cells, ATLL is a clonal expansion of CD4+, CD3+ and CD25+ T-cells.

The downstream effectors pose another level of complexity during the inflammatory response. Many of them act as double edged swords with beneficial and detrimental effects, depending on the physiological environment and magnitude of expression [33]. They are mainly involved in fighting infection and inflammatory conditions. However in pre-neoplastic cells in which the apoptotic machinery has been compromised, the inflammatory downstream effectors may induce constitutive damage that drives the transformation process [34].

Inducible nitric oxide synthase (iNOS) is one of the most common downstream inflammatory effectors. It was found to be overexpressed in chronic inflammatory diseases as well as in various types of cancer [33-45]. iNOS is an enzyme catalyzing the production of nitric oxide (NO), which is an important regulatory molecule in both inflammation and cancer development [46-48]. NO is the precursor of the highly reactive nitrogen species peroxynitrite (ONOO-), an obligatory factor of oxidative and nitrosative modifications of DNA and proteins [49-52] (Figure 2). It has recently been shown that selective inhibitors of iNOS, that reduce the release of nitric oxide in vivo, inhibited the progression of tumorigenesis in several cancers models [50,53-57]. Moreover, the inhibition of lipopolysaccharide (LPS) induced NF-kB activation inhibits iNOS expression and NO production, and inhibits inflammation-mediated tumorigenesis in mouse models [58-65].

In contrast to other constitutively expressed isoforms, iNOS is only expressed in response to inflammatory cytokines such as TNF-α and IL-1β and transcriptional activators, such NF-kB [48,66-69]. In chronic inflammation models and inflammation-related tumorigenesis, iNOS may be persistently stimulated by cytokines and NF-kB activation in the tumor microenvironment. iNOS/NO signaling can also induce cyclo-oxygenase-2 (COX-2), which is another link between inflammation and cancer [59,70]. In view of the diverse effects of iNOS-produced NO, it is important to determine how cells regulate their iNOS/NO system. Nevertheless, the progressive alterations of DNA and protein modifications are probably the major outcome. In the present review, we will focus on these two functions of NO in the context of HTLV-1 induced leukemia, a representative human malignancy for which the etiological agent is clearly identified.

Expression of Nitric Oxide Synthases

Three isoforms constitute the family of nitric oxide synthases (NOS) that catalyze the production of nitric oxide (NO) from L-arginine [69,71,72]. Neuronal nitric oxide synthase (nNOS or NOS1) and endothelial nitric oxide synthase (eNOS or NOS3) are constitutively expressed at steady state in the corresponding tissues, and are involved in neurotransmission and vasodilation, respectively [69]. In contrast, inducible nitric oxide synthase is expressed de novo, in response to inflammatory mediators, and its expression varies depending on the physiological environment and the magnitude of the inflammatory response [73,74]. While nNOS and eNOS catalyze low levels of NO synthesis in a Ca2+ dependent manner, iNOS generates high levels of NO independent of Ca2+ [75]. iNOS expression has also been detected in a wide array of cells and tissues, including pulmonary and colonic epithelium, and hepatocytes, but the immune cells, mainly macrophages and neutrophils, are considered as the major sources of iNOS synthesis [36,76-86]. They generate large amounts of NO in the extrinsic environment, with a primary microbiocidal activity. iNOS can also be induced and expressed in virally-infected lymphocytes, specifically T cells, but little is known about its intrinsic effects in activated regulatory CD4+ T-cells [87-91].

While all nitric oxide synthetases enzymatically catalyze NO production, they only share 50% amino acid sequence similarity, and are located on different chromosomes [69,76]. Nitric oxide synthetases also differ by the mechanisms regulating their expression. Despite the fact that post-transcriptional, co-translational, and post-translational regulation play roles in NOS expression, the predominant regulatory mechanism is transcriptional regulation [71,72,80,92,93].
mechanism of transcriptional regulation of human iNOS is much more complex than those mediating the expression of constitutively expressed nNOS and eNOS, as well as the expression of murine iNOS [94,95].

For almost two decades, murine macrophages were used as a main research tool for iNOS investigations. A combination of lipopolysaccharides (LPS) that activate the Toll-like receptor, and interferon-γ (IFN-γ) that activates the interferon type II response through JAK1/STAT1 pathway was sufficient to induce the expression of murine iNOS [96-101]. However, human iNOS involves a complex mechanism of transcriptional regulation that requires a mixture of cytokines and transcription factors. There is considerable evidence to suggest that many signaling pathways are involved in human iNOS expression that include IKK-IkB-NF-kB, JAK/STAT, PI3K-Akt, and MAPKs as well as the ubiquitin-proteasome degradation pathways. NF-kB, AP1, STAT1α, IRF-1, Oct1, C/EBPβ, ATF-2 and cAMP responsive element are specific transcription factors that have been described to interact and activate the human iNOS promoter [72,94,100,102-117]. iNOS expression can also be stimulated in hypoxic conditions, and HIF-1α is one of the transcription factors that participate in the induction of iNOS expression [118,119].

**Regulation of the Expression of Inducible Nitric Oxide Synthase in HTLV-1 Infected Cells**

Significant questions have been addressed to understand the effect of iNOS/NO on tumor biology. High expression of iNOS has been targeted by selective inhibitors in many animal cancer models, including colon, breast, prostate, bladder, skin, esophageal, and head and neck cancers, but the mechanisms that involve iNOS/NO in tumorigenesis are yet to be determined [120-135]. It is very important to clearly delineate the mechanisms of iNOS expression and NO production and their effects in the tumor microenvironment before designing prevention and therapeutic strategies that target iNOS/NO signaling. This is because iNOS/NO signaling has an extrinsic effect, with anti-infection and anti-tumoral actions mainly generated by macrophages and neutrophils, while its intrinsic effect has a tumor promoting activity within the infected/inflamed cells. Therefore, it is more relevant to explore iNOS/NO signaling in a virally-induced tumor like HTLV-1 induced leukemia, in which the infected cells represent the appropriate recipient for investigating the intrinsic effect of iNOS/NO.

HTLV-1 induced leukemogenesis in CD4+ T-cells is mediated by the expression of the viral oncoprotein Tax [6,16,23,136-140]. Among its many oncogenic functions, Tax induces a potent inflammatory response through activation of the NF-kB pathway. Because Tax has an intermittent expression during the early stages of infection and it is rarely measurable during the acute phase of the disease, it is still not clear whether Tax exerts different functions between these two phases of virally-induced tumorigenesis [6,138,141]. In fact, Tax follows the approach of “hit and run”, in which it promotes the oncogenic events during the infection course and it hides to prevent its elimination by the immune CTL response. Although Tax induces irreversible events that lead to the transformation of HTLV-1 infected lymphocytes, its expression might still be required to maintain the ATLL malignancy at later stages [138]. Most likely, Tax drives the initial events of immortalization in HTLV-1 infected cells through the expression of inflammatory mediators by NF-kB activation. In fact, Tax transgenic mouse model develop an ATLL like disease, and express high levels of TNFα, IL-1β, IL-6 and IFNγ, which participate in iNOS expression[140,142]. Moreover, we and others have demonstrated high levels of iNOS expression in HTLV-1 infected cells and this activation required the NF-kB pathway [87,143,144].

We recently reported a comprehensive study, in which we investigated the mechanism of iNOS expression in HTLV-1 infected T-cells (Figure 3). By using a human iNOS promoter reporter and small interfering RNA, we demonstrated that an activation of the classical NF-kB pathway was sufficient to activate iNOS expression by HTLV-1 Tax, and this activation involved a combination of both NF-kB and JAK/STAT pathways [87]. Moreover, we reported an increase of the active phosphorylated form of the signal transducer and activator of transcription, p-STAT1, and the interferon regulator factor 1, IRF-1 in HTLV-1 and Tax expressing cells. We also demonstrated that JAK1/STAT1 activation by type II interferon, but not type I interferon, is required to induce iNOS expression. In addition to NF-kB transcription factors, p-STAT1 and IRF-1 have previously been reported to activate iNOS expression in chronic inflammation models [145-147]. Interestingly, the inhibition of the JAK/STAT pathway by ruxolitinib, a JAK1/JAK2 inhibitor reduced iNOS expression and NO production. Although JAK/STAT activation is intended to induce an interferon-dependent, antiviral response, its role in HTLV-1 transformation was not well defined [148]. Interestingly, a recent study showed that a combination of ruxolitinib and navitoclax, a Bcl-2/Bcl-xl inhibitor, dramatically lowered tumor burden and prolonged survival in an ATL murine model [149]. This combination strongly blocked ex vivo proliferation of ATL patients’ PBMCs, indicating that JAK/STAT pathway promotes the proliferation of HTLV-1 infected cells.

![Figure 5: S-nitrosylation of Key proliferative proteins – In addition to the well-characterized NO function as a signal transducer, S-nitrosylation, which is a covalent addition of NO to cysteine, has emerged as a major post-translational modification of proteins. Over the past decade, the number of substrates modified by S-nitrosylation has considerably increased to include the small GTPase Ras, the protein kinase AKT, and the phosphotase and tensin homolog PTEN, which are all examples of proteins involved in proliferation, cell survival and tumorigenesis. S-nitrosylation was found to be a major player in their respective functions.](image-url)
Whether iNOS/NO signaling induces and/or maintains the immortalization process in HTLV-1 infected cells is not clear. But, the establishment in vitro of newly infected PBMCs with an HTLV-1 infected cell line showed that new infection was associated with an increase of iNOS expression, suggesting that de novo expression of iNOS is induced with productive infection. The high level of expression of iNOS, which is detected in HTLV-1 transformed cell lines and in ATLL patients' samples, also suggest that iNOS/NO signaling is required to maintain the phenotype of HTLV-1 induced leukemia [87].

**Origin of Genomic Instability in HTLV-1 Infected Cells**

Genetic instability is defined by increased rates of DNA damage and by the inability to maintain a faithful integrity of the genome within the infected cells. Although DNA damage is continuously created by multiple sources, cells have evolved mechanisms that include tumors suppressors, cell cycle checkpoints, and DNA repair pathways to control genomic integrity. Consequently, DNA damage is repaired, or if it is left unrepaired, the cell will activate apoptosis.

In HTLV-1 infected cells, the origin of genetic instability was associated with defects in different DNA maintenance mechanisms: i) Amplification of centrosomes, a common cause for improper distribution of chromosomes and aneuploidy, which are two hallmarks of malignant cells. ii) Inactivation of cell division checkpoints, such Anaphase-promoting complex (APC) and mitotic spindle checkpoint (MSC) proteins, such MAD1 and MAD2, lead to chromosome missegregation and accumulation of multinucleated cells, which is a common phenotype of ATL cells. iii) Induction of DNA double strand breaks (DSBs) and inhibition of DNA damage repair pathways are also responsible for genetic defects in HTLV-1 infected cells. While other HTLV-1 viral proteins have been involved in the regulation of genomic instability, the Tax oncoprotein was described as the main modulator of these defects. DSBs are the most detrimental forms of DNA damage because they pose problems for replication, transcription, and chromosome segregation, and are often the origin of mutations found in malignant cells. DSBs were found increased in HTLV-1 and Tax expressing cells, suggesting that Tax is able to induce DSBs and inhibit DNA damage repair response. The majority of these DSBs were recently attributed to the high levels of iNOS-produced NO in HTLV-1 infected cells. In fact, the inhibition of NO production can reduce, suggesting an attenuation of DNA damage in HTLV-1 infected cells.

**Figure 3: Model of Tax-Mediated Genetic Defects - HTLV-1 induced leukemogenesis is mediated by the expression of the viral oncoprotein Tax. Among its many oncogenic functions, Tax induces DNA Double Strand Breaks and inhibits DNA repair responses, which are two mechanisms at the origin of genetic defects. DSBs, which are the most detrimental forms of DNA damage, are found to be induced in HTLV-1 and Tax expressing cells by iNOS-produced NO and this induction required an activation of NF-kB and JAK/STAT, p-STAT1, IRF-1 signaling pathways.**

**iNOS/NO and DNA Oxidation/Nitration**

Initial mutations in pre-neoplastic cells are believed to be induced by DNA oxidative and nitrosative molecules, such as reactive oxygen species and reactive nitrogen intermediates. NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) produces the superoxide O₂⁻, and iNOS utilizes L-arginine to generate NO. Both superoxide and nitric oxide are unstable molecules. However, the reaction between O₂- and NO generates a much more stable and highly reactive molecule, peroxynitrite ONOO-. ONOO⁻ is an obligatory factor for oxidative and nitrosative modification of DNA and proteins. DNA oxidative/nitrosative damage is seen as modification of deoxyribonucleic acid, and it mostly occurs on guanine (G) because of its high oxidation/nitration potential relatively to cytidine, thymidine, and adenine (Figure 4). The hydroxyl at the C8 position of guanine is oxidized to generate 7,8-dihydro-8-hydroxyguanine (8-OHG), which forms 8-oxo-dihydroguanine (8-oxo-dG) or the ring-opened 2,6-diamino-3-formamido-4-hydroxy-pyrimidine (FapyG), two of the most abundant oxidative DNA adducts [150-152]. In order to be repaired, the oxidized guanine, 8 hydroxyguanine (8-oxo-dG), or the nitrated guanine (8-nitro-dG) (Figure 4) must be removed by a specific DNA glycosylase and repaired as a single strand mutation by base or nucleotide excision repair (BER or NER) (for an excellent review, see ref [150]). If it is not repaired, the modified guanine will have preference to pair with an adenine during the synthesis of DNA. However, modifications on adjacent guanines of both strands, which is followed by excision of the modified nucleotides often creates double strand DNA breaks (DSBs) that will be repaired by one of the two most common DNA repair pathways, homologous recombination (HR) or the non-homologous end joining (NHEJ) [153-155]. If the type of DNA damage occurs during the DNA replication in the S-phase of the cell cycle, the DNA will be faithfully repaired by the HR DNA repair pathway. If DNA damage occurs at other phases of the cell cycle (G1 or G2), the DNA repair will be directed by the error-prone NHEJ pathway and will create permanent deletions in the genome [156,157]. Thus, these type
of DNA damage caused by oxidation that constitutes a danger to the affected cell is totally arbitrary [156].

Figure 4: NO and its derivative products oxidize and nitrate DNA and cause DNA modifications that are at the origin of DNA double strand breaks. Although all deoxyribonucleic or ribonucleic acids can be oxidized or nitrated, guanine (G) has the highest oxidation/nitration potential. Two of the most abundant oxidative DNA adducts are the 7,8-dihydro-8-hydroxyguanine (6-oxo-dG), and the 8-nitro-2′-deoxyguanosine (8-nitro-dG), which are generated by oxidation and nitration of the hydroxyl at the C8 position of guanine, respectively.

In chronic inflammatory models, we can imagine a scenario in which continuous mutations by oxidation/nitration are randomly created until a combination of selectively stable mutations have initiated the immortalization process [157]. Another set of mutations are probably required to switch on the transformation process. Consequently, the incidence of a given malignancy is random and it depends on the frequency of mutations incurred during an oxidative exposition. Here, it is important to analyze the mutation signature of different cancer models to understand this phenomenon, in which lung carcinoma and melanoma are incurring the highest rate of mutations because they are exposed to the maximum oxidation and ionization factors [158]. In fact, the oxidative/nitrosative damage is a continuous process that does not stop once transformation of a clone of cells is switched on. This is perceived by the presence of heterogeneous clones in cancers [159] in vivo, and by continuous expression of oxidative agents in transformed cell lines in vitro [87].

iNOS/NO and S-nitrosylation of Proteins

NO is also a major source for protein S-nitrosylation, a covalent modification of cysteine thiol. The list of proteins modified by S-nitrosylation is currently gaining more attention [160] because emerging data has shown a role of S-nitrosylation in multiple pathways important for tumorigenesis [62,161-165]. NO, specifically generated by iNOS, is extremely important for S-nitrosylation, and its resultant signaling pathways. Experimental data elucidated a correlation between iNOS-mediated nitrosylation and an aggressive tumor phenotype for breast cancer, lung cancer, colon cancer and prostate cancer [166]. The small GTPase Ras, the protein kinase Akt, and the phosphatase and tensin homolog PTEN are all examples of proteins involved in proliferation, cell survival and tumorigenesis. S-nitrosylation was found to be a major player in their respective functions.

Modification of a single conserved cysteine residue in the small GTPase Ras (Cys118 in human H-Ras) was one of the earliest described targets of S-nitrosylation [166]. This modification stimulates guanine nucleotide exchange and downstream pathways, including activation of mitogen-activated protein kinase signaling (MAPK). Recent findings showed that iNOS expression promotes tumorigenesis in ER-negative breast cancer by a mechanism in which NO induces S-nitrosylation of wild-type Ras, leading to phosphorylation and activation of the transcription factor Ets-1 through the Ras/MEK/ERK pathway [166]. Interestingly, the Ras protein has been shown to be involved in the survival of HTLV-1 infected cells [167,168]. Akt is another multifunctional regulatory protein that is also involved in cellular metabolism, proliferation and survival. Recent discoveries showed that Akt kinase activity was augmented when it was nitrosylated by iNOS at cysteine 224 [169,170]. Why iNOS differs from other NOS isoforms is still unclear. However, it is likely to be linked to a specific stimulus that only targets iNOS activation. PTEN, one of the main phosphatases regulating Akt dephosphorylation, is selectively S-nitrosylated by low concentrations of NO at a specific cysteine residue (Cys-83). S-nitrosylation of PTEN inhibits its activity and stimulates Akt activation [170] (Figure 5).

The dysfunction of Akt and PTEN was extensively studied in HTLV-1 infected cells [137,171-173]. Whether S-nitrosylation of these proteins has an effect on HTLV-1 induced leukemogenesis was not investigated. In the light of the new published data, it is important to characterize the S-nitrosylation modification of Ras, Akt and PTEN in those cells, and to functionally test the influence of S-nitrosylation on the signaling pathway of Ras, Akt and PTEN. Inhibition of NO production by inhibitors, or by shRNA, the use of Tax mutants defective in iNOS activation, or the use of Ras C118S, Akt C224S or PTEN C83S mutants should be investigated to determine the role of S-nitrosylation on the oncogenic activities of these proteins in HTLV-1 infected cells.

iNOS/NO as a Marker for Diagnosis and Treatment

Nitric oxide is an important cellular signaling molecule involved in many physiological and pathological processes. The NO synthesized in the endothelial and neuronal tissues induces vasodilatation and neurotransmission actions, respectively. However, NO generated by iNOS in the immune cells has a central role in fighting infections, and it can be extremely mutagenic in chronically infected cells. The genetic alterations induced by NO are important requirements for induction of malignancy. iNOS-produced NO seems to play a critical role in cancer development because it was detected in various cancers and inhibitors targeting iNOS in animal models dramatically reduced tumorigenesis. Thus, iNOS/NO signaling can be considered as a novel and potential therapeutic target and iNOS/NO measurement can serve as useful assays in providing diagnostic for potential malignancies. Further experimentation on iNOS/NO signaling is required in order to develop new strategies for cancer prevention and treatment.

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