The Molecular Biology of the Myeloproliferative Leukemia Virus

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Introduction

Most acutely transforming leukemogenic or sarcomagenic retroviruses have transduced in their genomes altered cellular genes named oncogenes [1]. These viruses are usually defective in replication as a result of the deletion of structural genes, and they require a helper virus for propagation. Isolation of new acutely transforming retroviruses, comprehensive studies of their physiopathological processes, and molecular analysis of their genomes remain, therefore, powerful means for the identification of key genes involved in the regulation of cell growth, differentiation, or development.

The purpose of this paper is to summarize the data that we have accumulated over the past few years on a recently isolated acute leukemogenic murine retrovirus named myeloproliferative leukemia virus (MPLV).

Isolation of the MPLV

We isolated the MPLV in 1985 at the Curie Institute (Orsay, France) during a research program designed to evaluate the in vivo transforming properties of different Friend helper viruses (F-MuLV). While several clonal F-MuLV isolates have the capacity to induce a rapid erythroblastosis in newborn-inoculated NIH Swiss or BALB/c mice [2], DBA/2 mice were found to be resistant to this early erythroleukemia [3]. Nevertheless, they developed various types of hematopoietic malignancies after a latent interval of 7–12 months [4–6]. In general, these leukemias (either myelogenous, lymphoid, or erythroid) were associated with a more or less severe anemia.

Out of 238 DBA/2 mice inoculated at birth with F-MuLV clone 57 [7], one mouse developed, after 7 months of infection, an hepatosplenomegaly unusually accompanied with a polycythemia. Cell-free extract prepared from the original leukemic spleen or supernatant medium from an in vitro permanent cell line derived from the leukemic spleen cells caused an explosive leukemia upon inoculation into adult mice of most strains, including C57Bl strains. The disease was characterized by hepatosplenomegaly, polycythemia, pronounced myelemia but no thymus or lymph node involvement, and death within 1–3 months. Spleen and liver were extensively infiltrated with maturing precursor cells belonging to the granulocytic, erythroblastic and megakaryocytic lineages. Typically, the blood of severely diseased animals was also massively invaded by morphologically normal polymorphonuclears, erythroblasts, and platelets.

Several hematopoietic lineages were obviously involved in this disease, hence our name for the virus isolate, "myeloproliferative leukemia virus" [8].

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Genetic Analysis of MPLV Isolate

Virologic studies of MPLV by Penciolelli et al. [9] demonstrated that this highly leukemogenic virus isolate contained two dissociable retroviral genomes: one was the parental replication-competent F-MuLV 57, and the second was a new replication-defective component now designated as MPLV. A comparison of viral RNA species expressed in F-MuLV alone or F-MuLV + MPLV-producing cells by Northern blot analysis showed that MPLV was 0.8 kb shorter than F-MuLV and that a deletion had probably occurred in the MPLV *env* gene. This was further confirmed by the establishment of the MPLV restriction endonuclease map which was compared with that of F-MuLV [10]. From their data, these investigators concluded that the MPLV-defective genome

(a) was derived from F-MuLV,
(b) had conserved the F-MuLV *gag* and *pol* regions, and
(c) was deleted and rearranged in its *env* region [9].

Although MPLV does not transform fibroblasts in culture, its isolation free of replicating F-MuLV in nonproducer cells was feasible since the MPLV titer in the original isolate was approximately equivalent to that of F-MuLV. By the technique of limiting dilution and single-cell cloning, nonproducer cells containing MPLV were derived from *Mus dunni* fibroblasts [9]. Supernatant medium from these nonproducer cells did not cause any disease in inoculated mice demonstrating the defectiveness of MPLV. However, when superinfected with a variety of replicating helper viruses, supernatants reproduced the same acute myeloproliferative syndrome as caused by the original isolate. These experiments provided circumstantial evidence that the helper-dependent MPLV genome contained the genetic information necessary for the observed pathological processes.

Genomic Composition of MPLV

In an attempt to define the origin and nature of the genetic sequences contained in the MPLV-rearranged *env* region, Souyri et al. [11] derived cDNA probes which were nonhomologous to sequences contained in F-MuLV. Two probes were found to be MPLV specific, in that they hybridized to RNA of MPLV-containing nonproducer cells but did not hybridize to RNA of ecotropic MuLVs nor to RNA of amphotropic or xenotropic murine viruses. This indicated that, in contrast to Friend spleen focus-forming viruses (SFFV), MPLV did not result from a recombination between F-MuLV and a portion of the *env* gene of murine xenotropic virus [12, 13].

A full-length biologically active MPLV provirus was molecularly cloned from a genomic library of a nonproducer *Mus dunni* clone [11]. Sequence analysis revealed that the MPLV *env* gene contains a large open reading frame which could code for a polypeptide of 284 amino acids. This protein would contain 64 amino acids derived from the amino terminus of the F-MuLV gp70, including the signal peptide, 36 amino acids from a central region of the F-MuLV *env* gene, and 184 amino acids that are specific to MPLV (Fig. 1). A hydrophobicity plot of the amino acids sequence revealed that, in addition to the 34 hydrophobic amino acids of the gp70 signal peptide, the MPLV-specific domain contained a stretch of 22 uncharged amino acids. Thus, the putative MPLV *env* product presents the features of a transmembrane protein comprising an extracellular domain of 143 amino acids, a single transmembrane domain of 22 amino acids, and a cytoplasmic domain of 119 amino acids without consensus sequence for kinase activity [14]. Computer analysis of the deduced amino acid sequence revealed that the MPLV-specific sequence did not correspond to any known genes.

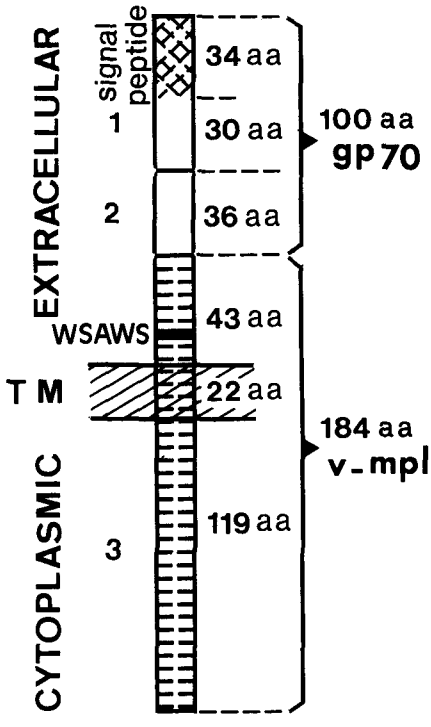

Fig. 1. Schematic representation of the putative MPLV *env* product. The ENV-*mpl* fusion protein consists of three fragments: part *1* derives from the NH_2-terminal region of the F-MuLV envelope gene (gp70) and contains a signal peptide; part *2* derives from a central part of the F-MuLV gp 70; part *3* corresponds to a transduced cellular sequence, most probably truncated at its N-terminus. The product encoded by this rearranged gene has the features of a single membrane-spanning domain (TM). The extra cellular domain of v-*mpl* possesses the amino acid sequence WSAWS highly conserved in the hematopoietin receptor superfamily, while the cytoplasmic domain does not contain consensus sequence for know catalytic activity

MPLV Has Transduced a Novel Oncogene

Since nonviral sequences found in the genome of acutely transforming retroviruses derive from cellular genes and are conserved phylogenetically, we looked for the presence of MPLV-specific sequences in genomic DNA from different mammals. Under stringent hybridization conditions, discrete bands were revealed in DNAs from mouse, rat, mink, dog, cow, and human. In addition, MPLV-specific probes recognized a 3.0-kb mRNA in spleen and bone marrow from adult mice and in fetal liver cells, but not in nonhematopoietic tissues [11]. Thus, taking into consideration the biological properties of MPLV, the cellular origin of the sequence contained in its *env* gene, the conservation in the genome of mammals and the expression in normal hematopoietic tissues, we concluded that MPLV had transduced a novel oncogene which was designated as v-*mpl*. By in situ hybridization and genetic analysis studies, chromosomal localization of the c-*mpl* proto-oncogene was assigned to mouse chromosome 4 (Vigon et al., unpublished data) and to human chromosome 1p34 [15].

Leukemogenic Properties of MPLV

In vivo studies by Wendling and coworkers have indicated that MPLV induced a rapid suppression of growth factor requirements for in vitro colony formation of a large spectrum of committed as well as multipotential progenitor cells [16, 17]. The primary manifestation of viral infection was a switch to erythropoietin (EPO) independence of the colony forming unit–erythroid (CFU-E) population which was complete in the spleen after 6 days of infection. A possible stimulating effect of EPO present or secreted in the culture medium was ruled out by the addition of neutralizing anti-EPO antibodies to the culture system. The effects of MPLV infection on the early and primitive erythroid progenitor cells (BFU-E) was assessed in methylcellulose serum-free cultures. It was found that well hemoglobinized pure and mixed erythroid colonies developed without the addition of interleukin-3 or EPO. Moreover, while a majority of colonies contained erythroblasts mixed with megakaryocytes, about 12% revealed three or more lineages of differentiation [16]. Further in vivo studies have documented that MPLV infection also induced the spontaneous colony formation of myeloid progenitors, i.e., granulocyte macrophage colony-forming cell (GM-CFC) granulocyte (G)-CFC, mega-

karyocyte (Meg)-CFC, and mixed CFC, probably as a result of direct infection of these progenitors and not as a consequence of a paracrine secretion of soluble colony stimulating factors by the accessory cells [17]. These observations supported the conclusion that MPLV acts on various progenitors, inducing their proliferation and terminal differentiation independently of signals normally provided by colony stimulating factors, interleukins, EPO, or any conditioned medium.

However, formal proof that MPLV can transform hematopoietic target cells in the absence of coinfection with a replicating MuLV was not provided by these experiments. We addressed this question by producing helper-free MPLV stocks using the packaging psi-CRE cell line that produces a high titer of infectious, nonreplicating particles but does not yield helper virus [18]. When adult ICFW mice were intravenously given helper-free preparations of MPLV, more than 90% of the mice were healthy 2 months after inoculation. Nevertheless, we observed that MPLV induced a mild but transient spleen enlargement with the appearance of colonies well visible on the spleen surface on days 5, 10, and 15 after inoculation. Histologically, colonies were composed of erythroblasts, or erythroblasts, granulocytes, and megakaryocytes clustered together in the splenic red pulp. On day 25 and thereafter, these colonies disappeared, leaving spleens with a normal aspect. In contrast, when helper-free preparations of MPLV were injected into mice pretreated with the aplastic drug 5-fluorouracil (5-FU, 150 mg/kg body weigth, 4 days before virus inoculation), all animals developed a typical MPLV syndrome and died from overt leukemia within 2 months (Wendling et al., unpublished data).

Together these data indicate that

(a) the MPLV component is primarily responsible for the myeloproliferative effects of the viral complex,
(b) expression of MPLV in erythroid and myeloid progenitors abolishes their growth factor requirement for in vitro colony formation, and
(c) induction of leukemia occurs in 5-FU-pretreated mice, suggesting that stable infection of cycling primitive progenitors is critical for leukemia development.

In Vitro Transformation Properties of MPLV

An area of current research in our laboratory is related to the ability of a helper-free preparation of MPLV to transform hematopoietic cells in vitro. A 2-h incubation of bone marrow cells enriched in highly dividing primitive progenitors by treatment of mice with 5-FU was sufficient to induce autonomous colony formation of about 30% of the colony-forming cells present in the preparation. Cytologically, half of these spontaneous colonies were composed of either granulocytes, megakaryocytes, or erythrocytes, while the remainders were mixed colonies of which about 20% contained three or more lineages of differentiation. Upon replating, the multilineage colonies produced secondary and tertiary mixed colonies, suggesting self-renewal [11].

The question of whether or not transformation of hematopoietic progenitors would lead to the generation of immortalized cell lines was then investigated. When marrow cells were cultured in liquid medium, it was observed that rapidly dividing nonadherent cell populations were produced in MPLV-infected cultures. After 10 to 12 days, these nonadherent populations could be transferred into fresh flasks devoid of stromal feeder layers. Cells continued to proliferate and generated permanent suspension cultures containing polymorphonuclears, megakaryocytes and erythroblasts. Upon continuous passages, the majority of the cell lines evolved towards a more restricted phenotype which remained stable over several months. Diverse immortalized

megakaryocytic, myelomonocytic, erythroblastic, or mastocytic cell lines retaining the ability to differentiate could easily be obtained. Since these permanent cell lines evolved from a multipotential to a more restricted phenotype, we investigated whether they were polyclonal or monoclonal by studying proviral-cell DNA junctions. Cultures were polyclonal 5 days after initiation. However, after 3 weeks and at a time where all cultures displayed a multipotential phenotype, one or a few major proliferating clones were detected in each cell line. Interestingly, the same clones were still found after 3 months of continuous passages when the cell lines appeared to be restricted in their differentiation potential [11]. Thus, it seems likely that MPLV induces the clonal outgrowth of a single or few transformed, probably multipotential, stem cells (clonal selection), the full differentiation capabilities of which being lost along with continuous culturing (clonal evolution).

The obtaining of immortalized in vitro cell lines raised the question of whether cells were tumorigenic. To approach this problem, 2×10^6 cells were subcutaneously grafted into either syngeneic or nude mice. Upon repeated assays, none of the cell lines developed tumor nodules at the site of inoculation when cells from cultures less than 4 months old were grafted. After prolonged passages (more than 7 months), 60% of the cell lines produced hematopoietic subcutaneous tumoral nodules, suggesting that additional genetic events must have occurred to reach a full malignant state.

Summary and Current Knowledge

The myeloproliferative leukemia virus isolate consists of two distinct viral components: a replicating F-MuLV and a helper-dependent MPLV. MPLV accounts for the rapid in vivo and in vitro transformation of a broad spectrum of multipotential, myeloid, and erythroid progenitors which acquire growth factor-independent proliferation and differentiation. By sequence analysis of a biologically active clone, MPLV has been shown to be an *env* recombinant virus containing sequences derived from the F-MuLV *env* gene and additional nonviral cellular sequences. These nonviral sequences are conserved in various mammals and are expressed in hemopoietic tissues from normal mice. MPLV was thus generated by transduction of an oncogene (v-*mpl*) in the envelope region of an F-MuLV genome. v-*mpl* does not correspond to any known gene, but the putative MPLV *env* fusion product has the features of a transmembrane protein with the N-terminal signal sequence of the F-MuLV gp70 directing the polypeptide across the membrane and a single transmembrane domain. Interestingly, the extracellular domain of v-*mpl* possesses, 13 amino acids upstream to the membrane-spanning domain, the amino acid sequence WSXWS, highly conserved in all cytokine receptors that make up the hematopoietin receptor superfamily [19]. In addition, a significant number of conserved amino acids were found when the extracellular domain of v-*mpl* was aligned with that of the IL-2β, IL-3, IL-4, IL-6, IL-7, GM-CSF, G-CSF, and EPO receptors [11]. Since the N-terminal part of the fusion protein consists of F-MuLV-derived sequences, it is not yet known whether the c-*mpl* proto-oncogene product would contain the highly conserved cysteine residues characteristically found in the ligand-binding domain of each of these receptors [19]. Nevertheless, with regard to the general features of v-*mpl*, it is tempting to speculate that MPLV has transduced a truncated form of a putative cytokine receptor. Cloning of the proto-oncogene cDNA is currently underway in our laboratory to allow further comparison.

A major focus of future research will be to understand the mechanism by which this viral oncogene can short-circuit the growth-regulatory signals delivered by the binding of various hematopoietic growth factors to their specific receptors.

This requires further studies on the mechanism of signal transduction by MPLV and by other receptors of the same family.

Acknowledgments. We thank Martine Charon, Laurence Cocault and Paule Varlet who provided excellent technical assistance. This work was supported by grants from the Institut National de la Recherche Médicale (INSERM), the Centre National de la Recherche Scientifique (CNRS), the Association pour la Recherche contre la Cancer (ARC), the Fondation pour la Recherche Médicale, and the Ministère de la Recherche et de la Technologie.

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