2 Molecular Biology and Cytokines

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1. INTRODUCTION

The proliferation and differentiation of hematopoietic cells is regulated by the interaction of multiple genes, transcription factors, and proteins.

The genetic information is encrypted in DNA. The cell synthesizes messenger RNA (mRNA) via transcription. The corresponding proteins are synthesized (translation) in the cytoplasm. Molecular biology is of special importance to the clinical hematologist, as leukemias and lymphomas are clonal neoplasms and can be detected by DNA analysis if a genetic marker lesion is known. The discovery of DNA sequences in normal cells that are homologous to RNA viruses led to the description of oncogenes. These genes, also called proto-oncogenes, are responsible for cell growth and differentiation in normal cells. In several neoplasms, a mutation or abnormal expression of oncogenes is present. The loss of antioncogenes also contributes to the initiation and progression of tumors. Other genes that permit the survival of malignant cells are the group of antiapoptotic genes. There is reasonable hope that the elucidation of the molecular pathogenesis of leukemias and lymphomas will lead to new treatment possibilities.

From: *Contemporary Hematology: Modern Hematology, Second Edition*
Edited by: R. Munker, E. Hiller, J. Glass, and R. Paquette

Cytokines are proteins involved in cell-to-cell communication. They are produced and secreted by both normal and malignant cells. Some cytokines have growth-stimulatory properties; others inhibit cell growth or induce cell death. Most cytokines bind to specific receptors on the cell surface. The number of known cytokines and related molecules has expanded enormously in recent years. Some cytokines, like the family of interferons (IFNs), the family of colony-stimulating factors (CSFs), and interleukin (IL)-2 have entered into clinical practice. It can be foreseen that in the next years, even more cytokines and cytokine antagonists will become important in clinical hematology.

2. RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology has made possible the isolation and characterization of genes from the human genome. This method involves the use of enzymes (restriction endonucleases) that recognize and cleave double-stranded DNA at certain points. In addition, other nucleic acid-modifying enzymes and plasmids (extrachromosomal DNA from bacteria) enable further re-engineering of DNA. This can be done, for example, by inserting the desired DNA sequences into these plasmids, expanding them in vitro, and then retrieving large quantities of recombinant proteins in eukaryotic cells. Any tissue can be examined for the presence of certain genes by Southern blotting. Minute amounts of DNA can be amplified with PCR and then visualized on gels. In Northern blots, RNA is separated on gels and then specific mRNA is detected after hybridization with cloned DNA fragments. In Western blots, the presence and molecular weight of proteins in cell extracts or body fluids is examined by immunological methods.

3. CLONALITY OF TUMORS

The clonality of human tumors is one of the major principles underlying malignant transformation. Clonality means that a tumor is derived from a single transformed cell (and retains some characteristics of this cell). Usually, it takes several years until a tumor has grown from a single cell to the stage where it can be diagnosed clinically. Virtually all hematological neoplasms (leukemias, lymphomas, and myeloproliferative and myelodysplastic syndromes) are clonal. One of the few exceptions is the lymphoproliferative disorder associated with the Epstein-Barr virus (EBV), which is clinically malignant, yet polyclonal in many cases. In some elderly persons, or in the immediate period after bone marrow transplantation, monoclonal or oligoclonal cells can be demonstrated in the absence of a malignancy.

The criteria for malignant growth are abnormal morphology, clinical aggressiveness, genetic changes, and clonality. The general rule is that all or most of these criteria should be present in order to diagnose a malignant tumor. There are

several methods to establish clonality in hematological disorders. Some of these methods are used only in research laboratories, whereas other methods have entered clinical routine.

3.1. Cytogenetics

The study of chromosomes, or cytogenetics, aids in the diagnosis of many human tumors and is a common method used to establish the clonality of abnormal cells found in the bone marrow, the peripheral blood, or any other tissue. The normal human somatic cell is diploid, meaning that each of the 22 chromosomes is present in two copies. In addition, the sex chromosomes, XX in females and XY in males, are present in all somatic cells. The cells of sperm or oocytes that have 23 single chromosomes are haploid. The normal human chromosomes are numbered 1–22 in decreasing order of size. Each chromosome has two arms, the shorter "p" arm and the longer "q" arm, which meet at the centromere. Chromosomes are usually visible only during metaphase. In order to study chromosomes, the cells are cultured in media and then arrested in metaphase with colchicine. Special stains are used to identify regions and bands of chromosomes. Common changes in chromosomes are gains and losses of chromosomes, translocations, deletions, and inversions. The genetic changes of pathogenetic or diagnostic relevance are not random, but involve all or most chromosomes studied; this establishes clonality. In a translocation, part of one chromosome is moved to another chromosome. A well-known translocation is t(9;22), where part of chromosome 9 is moved to chromosome 22 and vice versa. This translocation is also known as the Philadelphia chromosome and is diagnostic of chronic myelogenous leukemia (*see* Fig. 8.1). One refers to a deletion when part of a chromosome is missing. The deletion of part of a chromosome and its reattachment to the breakpoint in the opposite direction is called an inversion (denoted as inv). Sensitive cytogenetic methods can detect chromosomal changes in most leukemias and lymphomas. When even more sensitive molecular methods are used (*see* "Molecular Cytogenetics"), changes in the DNA of tumor cells can be demonstrated in virtually all hematological neoplasms. Some cytogenetic changes have prognostic relevance. Examples are t(15;17) and inv(16), which are markers for a better-than-average prognosis in acute myelogenous leukemia, and t(9;22), which is associated with an unfavorable prognosis in acute lymphoid leukemia. New cytogenetic aberrations often appear when a leukemia or lymphoma undergoes progression, as in the case of chronic myelogenous leukemias that acquire new cytogenetic abnormalities when entering the accelerated phase and then the blast crisis. The acquisition of new cytogenetic aberrations is called "clonal progression" or "clonal evolution" and is a poor prognostic sign. The analysis of chromosomes ("karyotyping") is usually done only with a limited number of cells (in routine cytogenetic analysis 15–25 metaphases are exam-

ined). Minor clones of aberrant cells can therefore be missed. Some cytogenetic aberrations are frequent in hematological neoplasms but not specific for a particular disease. For example, t(3;21) can occur in acute myelogenous leukemia as well as in the blast crisis of chronic myelogenous leukemia.

3.2. Molecular Cytogenetics

A further refinement in the area of cytogenetics has been achieved through the ability to clone the breakpoints for most translocations and other genetic changes in tumor cells. So-called fusion genes encoding novel RNAs and proteins are occasionally created by the genetic changes in tumor cells. An example is the *BCR-ABL* fusion gene resulting from the translocation t(9;22). The analysis of fusion genes can clarify certain mechanisms of transformation, as in the case of lymphoma cells with the translocation t(14;18), which express a protein inhibiting apoptosis. The most sensitive method to assay fusion genes is the PCR. PCR can be used for the study of minimal residual disease after intensive treatment or bone marrow transplantation, but PCR is not quantitative in routine use. With the same methods, point mutations or the expression of oncogenes can be assayed. The analysis of protein (Western blot), RNA (Northern blot), and DNA (Southern blot) is not as sensitive as PCR but permits an approximate quantification. The fraction of malignant cells that can be recognized by Western, Northern, or Southern blot is in the range of 1–5%. PCR can recognize one malignant cell in 10^5 normal cells if a specific molecular marker for the tumor cell is known. A new method known as fluorescence *in situ* hybridization (FISH) can also analyze nondividing interphase cells, allowing determination of the type of cell with a particular mutation or translocation. A disadvantage of FISH is that the currently available probes yield a rather high background. Molecular genetic methods such as PCR and FISH are intended as complements, not substitutes for, the classical cytogenetic methods. In order to perform PCR or FISH, the type of mutation must first be known.

3.3. Rearrangement of Immunoglobulin Genes or of the T-Cell Receptor

During the ontogeny of normal B-cells, the genes for the heavy and light chains of the different immunoglobulin molecules are rearranged sequentially. Similarly, the genes encoding the α, β, γ, and δ chains of the T-cell antigen receptor are rearranged in normal T-cells. In malignant B- or T-cells, the immunoglobulin genes or the T-cell receptor genes have clonal rearrangements. Again, these rearrangements can be studied by Southern blot (which is semiquantitative) or by PCR (which is the most sensitive method). The study of immunoglobulin gene rearrangements also detects cells at an early stage of B-cell development, but is not specific for the B-cell lineage, as acute myeloid leukemias occasionally have rearranged immunoglobulin genes.

3.4. Light-Chain Restriction

The analysis of light chains using flow cytometry or immunocytochemistry is a simple tool to detect clonality in neoplasms that express light chains (κ or λ) on their surface or in their cytoplasm. Mature lymphoid neoplasms (e.g., chronic lymphocytic leukemia or follicular lymphomas) express surface light chains, whereas tumor cells of multiple myeloma or some acute lymphoblastic leukemias express only cytoplasmic light chains. The normal ratio of κ to λ expressing B-cells in humans is about 2:1. If a significant imbalance of light-chain expression is found, clonality is likely.

3.5. Loss of Heterozygosity

Loss of heterozygosity (LOH) refers to the loss of genetic material from one allele at a specific gene locus. LOH is tested with PCR amplification of polymorphic microsatellite markers for a particular gene or locus. LOH is specific for the detection of clonally derived cells, but is not sensitive enough for the study of minimal residual disease. In addition, some normal persons have clonally derived cells that can be detected with LOH; therefore, control tissues should be studied. LOH studies as well as the study of the inactivation of genes on the X chromosome are only performed in specialized research laboratories.

3.6. X-Inactivation Assays

These studies are performed with genes located on the normal X chromosome. An example for such a gene is glucose-6-phosphate dehydrogenase (G6PD), which has two allelic forms, A and B. Normal females have two X chromosomes and may be homozygous for A or B or heterozygous (AB) in all somatic cells. Normal cells in heterozygote females are approx 50% of the A-type and 50% of the B-type. Using a simple enzyme assay for G6PD, it has been shown that myeloproliferative syndromes are clonal disorders involving myeloid, monocytic, and other cell types. These studies can only be performed in heterozygotes. More recently, the inactivation of other genes located on the X chromosome was studied by DNA- or RNA-based methods. An example is the gene encoding the human androgen receptor locus. Again, these studies can only be performed in females. The inactivation of the gene of interest on the X chromosome is studied by the analysis of methylation or transcription. An advantage of X-inactivation assays is that no prior knowledge of mutations or gene rearrangements is necessary. A caveat for these assays is that adequate controls are required because some individuals have a more or less than 50% likelihood of gene silencing on the X chromosome. Clonal cells can also be detected by this method in some older individuals without a malignancy being present.

3.7. Small RNA Molecules in Hematology

Recently, two types of small RNA molecules, microRNA (miRNA) and small interfering RNA (siRNA), have emerged as sequence-specific posttranscriptional regulators of both prokaryotic and eukaryotic gene expression. Below, a brief overview of miRNA and siRNA and their implications in hematological research is given.

3.7.1. miRNA

MiRNAs are a family of about 22 nucleotide small, functional, noncoding RNAs that have been shown to play important roles in various biological processes, including developmental timing, apoptosis, cell proliferation, fat metabolism and hematopoietic differentiation.

Hundreds of miRNA genes have recently been found in both animals and plants. In animals, miRNAs are transcribed by RNA polymerase II through sequential processing in the nucleus and cytoplasm. MiRNAs are initially transcribed as long transcripts (pri-miRNAs), which are cleaved by the nuclear endonuclease Drosha to generate the intermediate short, 60-70 nt hairpin structures (pre-miRNAs). Pre-miRNA is subsequently transported to the cytoplasm by exportin-5 and cleaved by RNase III Dicer to about 22 nt RNA duplex. One strand of the duplex is the mature miRNA, and miRNA regulates gene expression at the transcriptional and/or translational level.

One group reported the identification of more than 100 miRNAs from mouse bone marrow. Among them, three (miR-181, miR-223, and miR-142s) were specifically expressed in hematopoietic tissues. MiR-181 was preferentially expressed in B lineage cells. Overexpression of miR-181 by a retrovirus vector in hematopoietic stem cells led to an increase of cells in the B lymphoid compartment. The results indicate the potential role of miRNA in hematopoietic differentiation.

miRNA profiling in human B cell chronic lymphocytic leukemia (CLL) was performed by another research group. Significant difference in the expression pattern was observed between normal and CLL patient samples. More recently, it was discovered that the expression of miR-155 is increased in human B cell lymphomas. This observation has linked miRNA to the pathogenesis of human lymphatic malignancies.

In an in vivo model, “antagomirs” were created ablating endogenously overexpressed miRNAs. As a secondary phenomenon, multiple other genes were repressed in mice treated with “antagomirs.” Ultimately, “antagomirs” or similar molecules might be useful for treating human diseases including leukemia and cancer.

3.7.2. SiRNA

RNA interference is an evolutionarily conserved process of posttranscriptional gene silencing through siRNAs that mediate mRNA degradation. This phenomena was first described in C. elegans, and was found in different organisms such as plants, fungi, flies and mammals. DsRNAs are processed by the highly conserved RNase III Dicer and cleaved into 21-23 nt small interfering RNAs (siRNAs). siRNAs are then packaged into RNA-induced silencing complexes (RISC). The RISC directs the base pairing and cleavage of the target mRNA. In mammalian cells, dsRNA larger than 30 bp can also trigger interferon/antiviral response, leading to nonspecific silencing of cellular genes. The success of the direct introduction of chemically synthesized small duplex RNA into mammalian cells without triggering interferon response has allowed the development of many strategies to introduce siRNA. RNAi has been rapidly growing as an effective tool for study of gene function and for therapeutic applications.

Many hematopoietic genes have been targeted by siRNA, including BCR-ABL, AML1/MTG8, CD4 and CD8. Using a cell line bearing the BCR-ABL rearrangement, RNAi can achieve sequence-specific silencing of the BCR-ABL oncogene. Transfection of a 19 nt dsRNA specific for the BCR/ABL fusion mRNA into K562 leukemic cells depleted the corresponding mRNA and oncoprotein, and induced apoptosis. Reduction of BCR-ABL mRNA levels was also observed in primary CML cells upon introduction of anti BCR-ABL siRNA by electroporation. RNAi shows promise as a technology for future therapeutic development.

4. DIAGNOSTIC APPLICATIONS OF MOLECULAR BIOLOGY AND GENE THERAPY

A number of hematological diseases (e.g., thalassemias, sickle cell anemias, leukemias, and lymphomas) have a genetic basis, and the introduction of molecular methods has allowed for a more refined diagnosis of these disorders. For example, certain cases of chronic myelogenous leukemia were recognized that do not have the classical Philadelphia chromosome yet were shown by molecular analysis to harbor the *BCR-ABL* fusion gene. The classification of non-Hodgkin's lymphomas has been made more reproducible, as molecular and immunological methods have been employed for diagnostic purposes. The defective genes in hematological disorders may be inherited (germ-line defects) or acquired (somatic mutations). Because many of the molecular defects were defined, the replacement of defective genes by gene therapy is a logical treatment. For gene

transfer, human DNA sequences are introduced into a packaging system or vector (discussed in the following paragraphs) and transferred into the cell of interest. The vectors also often carry genes for antibiotic resistance, which allows later selection of transduced cells. The transduced cells synthesize proteins according to the DNA sequences transferred. Gene transfer can replace missing or defective genes or introduce genes usually not found in the target cells.

At present, human somatic gene therapy is at a turning point. It was shown for the first time that gene replacement could actually have major clinical benefit (normalizing the immune defect of children with X-linked severe combined immunodeficiency [SCID]). Almost at same time, the first major or serious complications were observed after gene therapy. A patient died from an infection or inflammatory response related to the adenoviral vector used for gene transfer. Out of 11 patients cured by gene therapy of X-linked SCID, two developed acute leukemia by insertional mutagenesis (*see* Chapter 10). These tragic events have tempered the enthusiasm that surrounded gene therapy and have led to additional safeguards for clinical protocols. However, basic research continues to make progress and new protocols are currently under development. Gene therapy can also promote an effective immunotherapy by making tumor cells immunogenic. The types of vectors used, some of the problems involved, and the first clinical applications of gene therapy are discussed in this chapter.

4.1. Viral Vectors and Gene Delivery Systems

4.1.1. Retroviral Vectors

This widely used modality of gene transfer has permitted the transfer of genes into cell lines, experimental animals, and patients. Retroviral vectors are based on the Moloney murine leukemia virus and can integrate only into dividing cells. The retroviruses used for gene transfer are made replication incompetent. Unfortunately, the efficiency of gene transfer is generally low and only 8–9 kb of foreign DNA can be transferred using these vectors. Furthermore, retroviruses integrate randomly into the host genome, which can theoretically cause insertional mutagenesis.

4.1.2. Adenoviral Vectors

Adenoviral vectors can also infect nonproliferating cells. These viruses are generally stable and can be purified. The uptake of adenoviral vectors is episomal; therefore, no long-term expression is achieved. Adenoviruses usually mediate a strong expression of the integrated genes. Up to 15 kb of foreign DNA can be transferred. The induction of antibodies that decrease the efficacy of repeated administration can cause a problem when using adenoviral vectors.

Adeno-associated virus is a defective parvovirus and can also be used in gene transfer experiments. This virus is nontoxic but difficult to prepare on a large

scale. Adeno-associated virus integrates into the host genome and potentially infects a wide range of target cells.

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4.2. Nonviral Vector Systems

Liposomes are a simple, nontoxic, and noninfectious modality of gene transfer. The efficiency of gene transfer, however, is rather low, as liposomes compete with serum in vitro and the DNA transferred in liposomes is degraded in the cell.

At present, further vectors based on viruses (e.g., herpesviruses and lentiviruses) are being developed. A common problem of all the currently used gene transfer systems has been that only a minority of cells can be infected and the expression of the transferred gene is lost after some time.

4.3. Clinical Gene Therapy Protocols

Gene therapy is an attractive idea for hematological disorders, in which stem cells can be treated, and for cancer in general, in which patients have limited options after relapse.

The first group of studies aims at the *replacement of missing genes*. A small group of children with adenosine-deaminase (ADA) deficiency has been treated by gene transfer. These patients have severely defective T- and B-cells (SCID). As a consequence of the deficient enzyme, adenosine and desoxy-adenosine triphosphate accumulate in cells, thus interfering with DNA synthesis. T-cells from children with ADA deficiency were infected in vitro with ADA-expressing retroviruses, then re-infused into the children after in vitro culture. The first children in which the ADA gene was transferred obtained about 25% of the normal ADA levels and had fewer infections than before. The administration of transfected lymphocytes has to be repeated every 3–6 mo. More recently, some patients underwent nonmyeloablative conditioning with busulphan prior to transplantation with oncoretrovirally transduced $CD34^+$ cells. As expected, a transient myelosuppression was observed, but otherwise the procedure was well tolerated. The transduction efficiency of clonogenic progenitors was between 21 and 25%. At preliminary analysis, the peripheral blood B-, T-, and natural killer (NK) cells showed levels of gene marking between 70 and 100%. The transfer of other genes is also under investigation. As mentioned, SCID could be success-

fully corrected by gene therapy. X-linked SCID is due to a defect in the common γc-chain of the IL-2 cytokine receptor family. Without specific treatment, SCID is fatal within the first year of life. Two groups (one in France, one in Great Britain) treated children with SCID by gene therapy.

In Gaucher's disease, several groups have transferred the gene coding for glucocerebrosidase into stem cells from bone marrow or blood. In Hurler syndrome, a storage disease, the first clinical trials with gene transfer into hematopoietic stem cells are being performed. In hemophilia, attempts are being made to replace the gene coding for factor VIII or IX by transducing skin or other tissues. Hemophilias are attractive disorders for gene therapy because a low level of transgene expression (1–5%) might offer major clinical benefit. Currently, the results of 6 phase I/II clinical trials are under evaluation. It appears that the vectors used had little toxicity but did not lead to any lasting transgene expression. More recently, gene therapy trials for hemophilia B were initiated using recombinant adeno-associated virus, which has not been associated with human diseases. Chronic granulomatous disease is another disease in which the genetic defects have been characterized and gene replacement has entered phase I clinical studies. Gene replacement studies also aim at replacing mutated genes in tumor cells, for example, by the replacement of the missing or mutated *p53* gene in solid tumors and the alteration of abnormal growth in leukemic cells that have a leukemia-specific translocation. The goal of reversing malignant growth can theoretically also be accomplished by downregulating the expression of oncogenes and certain growth factor genes with antisense oligonucleotides or small interfering RNAs.

The second group of clinical protocols are *gene-marking studies*. The aim of these studies is not to cure a disease but to follow the fate of transplanted or transduced cells in vivo. Small amounts of blood or other tissues are taken and the presence or absence of the marker gene is demonstrated with PCR. From these marker gene experiments, it was shown that relapse of acute leukemias after autologous bone marrow transplantation derives both from the host (residual leukemic cells surviving the conditioning) and from the graft (autologous stem cells contaminated with leukemic progenitor cells).

The third group of studies aims at stimulating a *local or systemic antitumor immunity* by transducing tumor cells with cytokine genes (e.g., the genes for granulocyte/macrophage colony stimulating factor [GM-CSF], IL-2, or tumor necrosis factor [TNF]). The transduced tumor cells are irradiated and then reinjected into the host.

In some animal models, "bystander effects" are observed whereby only a fraction of the tumor cells is transduced, but the subsequent immune reaction also eliminates or attacks nontransduced tumor cells. A similar approach is also taken

with tumor-infiltrating lymphocytes. These cells are genetically modified, irradiated, and then re-injected into the patient. A new development involves transducing tumor cells with peptide transcription units. The proteins synthesized by the transduced cells bind to class I human leukocyte antigen molecules and thereby can stimulate a T-cell-dependent immune response against the tumor. The modification of dendritic cells may also promote a potent cellular immune response directed against tumors. Many groups are focusing on the generation of antitumor vaccines aimed at making the immune system recognize hitherto poorly immunogenic tumor cells. Many tumor cells fail to express the co-stimulatory molecule B-7. If B-7 is transferred, the tumor cells can become immunogenic and can be eliminated by the immune system. In patients with AIDS, several groups are trying to restore cellular immunity either by immunizing asymptomatic patients with transduced cells or enhancing the degradation of HIV-mRNA by ribozymes, small interfering RNAs, or other mechanisms.

Finally, a diverse group of studies aims at modifying the general behavior of normal or malignant cells. An example is the transfer of a multidrug resistance gene to hematopoietic stem cells, thereby making them resistant to chemotherapeutic drugs. This procedure could permit the use of higher doses of drugs for the treatment of a malignant tumor, at least theoretically. The transfer of suicide genes can also be used to eliminate tumor cells. The first clinical application of a suicide gene was in patients with glioblastomas. Vectors coding for thymidine kinase were injected into these brain tumors. After integration, a drug (gancyclovir) then activated the suicide program of the transduced tumor cells. A similar approach has also been used in the adoptive transfer of T-lymphocytes. These cells are transduced with a suicide gene and then transfused into a recipient to induce a graft-vs-leukemia reaction. Once a significant graft-vs-host reaction has developed, the suicide program of the transduced cells is activated, thereby stopping any noxious effects.

5. CYTOKINES

5.1. Colony-Stimulating Factors

CSFs were originally defined as substances that stimulate the colony growth of blood cells in soft agar or methylcellulose. The first hematopoietic growth factors described were granulocyte colony-stimulating factor (G-CSF), GM-CSF, macrophage colony-stimulating factor (M-CSF), and IL-3. Accordingly, G-CSF supports the in vitro growth of granulocyte colonies, GM-CSF supports the growth of mixed colonies with both granulocytes and monocytes/macrophages, and M-CSF stimulates the growth of pure macrophage colonies. IL-3 promotes the growth of mixed colonies that may also contain red cell precursors and

megakaryocytes. In the meantime, additional cytokines or ILs have been described that also influence the differentiation of hematopoietic cells in vitro and in vivo.

In this section, we discuss the basic biology of CSFs and their clinical applications in hematological disorders.

5.1.1. Basic Biology

G-CSF is a glycoprotein with a molecular weight (MW) of 18 kDa, which can be synthesized by numerous cells, including monocytes, fibroblasts, and epithelial and endothelial cells, but not T-lymphocytes. Substances that induce the secretion of G-CSF are bacterial products or other cytokines.

GM-CSF is a glycoprotein with an MW of about 14–35 kDa, which is synthesized by mast cells, T-lymphocytes, endothelial cells, fibroblasts, and thymic epithelial cells. In vitro, bacterial products, other cytokines, and phorbol esters can induce the synthesis of GM-CSF.

M-CSF is also a glycoprotein, has an MW of about 45–70 kDa, and is synthesized by monocytes, macrophages, fibroblasts, epithelial and endothelial cells, and osteoblasts. M-CSF is also induced by other cytokines.

IL-3 has an MW between 14 and 28 kDa and is produced by T-lymphocytes and mast cells in response to mitogens, phorbol esters, calcium ionophores, and an immunoglobulin (Ig)E receptor activation.

The genes for most human CSFs are located on chromosome 5, with the exception of the G-CSF gene, which is found on chromosome 17.

The action of cytokines is mediated via high- and low-affinity receptors on the cell surface. These receptors are specific for each CSF; however, cross-modulations occur, for example, between IL-3 and GM-CSF. Most factors (G-CSF, M-CSF, and GM-CSF) not only support the proliferation and differentiation of progenitor cells, but also increase the functional capacity of mature cells (granulocytes and monocytes).

5.1.2. CSFs Licensed for Clinical Use

GM-CSF is available in the United States as a recombinant cytokine expressed in yeast (sargramostim). In other countries, GM-CSF is also available as a recombinant protein expressed in *Escherichia coli* (molgramostim). The recommended dose of sargramostim is 250 μg/m^2 (given by intravenous or subcutaneous injection). The indications for sargramostim approved by the Food and Drug Administration (FDA) are: promote recovery of myelopoiesis in patients with acute myelogenous leukemia (AML) older than 55 yr who received induction chemotherapy; mobilize autologous stem cells; promote myeloid recovery after autologous or allogeneic stem cell (bone marrow) transplantation; and improve myelopoiesis in patients with delayed engraftment after autologous (allogeneic) engraftment.

G-CSF is available in the United States as a recombinant protein expressed in *E. coli* (filgrastim). In other countries, G-CSF is also available as a recombinant protein expressed in a mammalian cell line (lenograstim). The recommended dose of filgrastim is 5 μg/kg (administered by subcutaneous or intravenous injection). The FDA has approved these indications for filgrastim: support myeloid recovery after chemotherapy for AML; mobilize peripheral blood stem cells; accelerate myeloid recovery after myelosuppressive chemotherapy or bone marrow or stem cell transplantation and severe chronic neutropenia. Because CSFs are expensive and in many instances shorten neutropenia but do not improve the ultimate prognosis of the underlying malignancy, the American Society for Clinical Oncology (ASCO) has developed guidelines for the use of CSFs after myelosuppressive chemotherapy. A primary prophylaxis is only recommended if the risk of febrile neutropenia is ≥40% per cycle of chemotherapy. If a patient has experienced febrile neutropenia during a previous cycle of chemotherapy, the use of myeloid growth factors appears justified to maintain dose-intensity in curable malignancies (testicular cancer, Hodgkin's and non-Hodgkin's lymphoma).

5.1.3. Use of CSFs in Patients With Hematological Disorders

The cloning of the genes for CSFs and the production of recombinant proteins has made large-scale clinical use of these cytokines possible. In contrast to other cytokines such as TNF and IL-2, the CSFs are generally well tolerated. Some patients treated with G-CSF experience bone pain, and have a slight enlargement of the spleen as side effects. Lethargy, bone pain, and slight fever are symptoms observed in some patients treated with GM-CSF and, especially at higher dosages, cases of phlebitis, generalized edema, and pericarditis were encountered (capillary leakage syndrome).

In vivo, the plasma half-life of the CSFs is in the range of minutes to hours. Subcutaneous administration results in a more sustained plasma level and possibly in a better clinical efficacy than intravenous bolus injections. The doses of CSFs tested in various studies until now have ranged between 1 and 30 μg/kg/d. G-CSF stimulates a dose-dependent leukocytosis (increase of neutrophils) in patients and in normal individuals, whereas GM-CSF also increases the number of eosinophils and monocytes. The action of the hematopoietic growth factors is reversible within days after discontinuing their administration.

The hemopoietic growth factors G-CSF and GM-CSF are widely used in patients with acquired neutropenia. G-CSF is especially well tolerated. According to most studies, the period of neutropenia can be shortened by several days in high-risk patients who have undergone autologous or allogeneic stem cell transplants or other high-dose chemotherapy, thus reducing the likelihood of infection. The general use of these factors in all patients who undergo high-dose

therapy is not recommended because of the high cost involved. Only patients who have a high likelihood of serious infections should be treated prophylactically. The increase in the dose of cytostatic drugs is limited not only by neutropenia, but also by other toxicities that cannot be ameliorated by the CSFs. The use of CSFs in patients with a high likelihood of infection also applies to other situations such as severe aplastic anemia. In summary, the use of CSFs in patients with acquired neutropenia is mainly supportive, but may improve the prognosis in high-risk situations.

A clear indication for the use CSFs is idiopathic neutropenia. In congenital neutropenia (Kostmann syndrome), for example, the patients suffer from septicemias, pneumonias, and other infections from early infancy. After treatment with G-CSF, the number of neutrophils normalizes and chronic ulcerations and infections heal. Several patients have now been treated for more than 15 yr with few side effects. GM-CSF is less effective in patients with Kostmann syndrome and increases more eosinophils than neutrophils. In cyclic neutropenia, G-CSF does not eliminate the cyclic variations of neutrophils, but greatly reduces infectious complications. Other acquired neutropenias (autoimmune or idiopathic) also benefit from hematopoietic growth factors.

5.2. Pegfilgrastim

Pegfilgrastim is filgrastim (G-CSF) bound to polyethylene glycol, which significantly increases the in vivo half-life compared with native recombinant G-CSF. Therefore, one injection of pegylated G-CSF per cycle of chemotherapy is sufficient to stimulate neutrophil recovery. The side effect profile is comparable to G-CSF. The indication of pegfilgrastim as approved by the FDA is to reduce the risk of neutropenia following myelosuppressive chemotherapy. The recommended dose of pegfilgrastim is 6 mg given by subcutaneous injection.

5.3. Thrombopoietin

Thrombopoietin (TPO) or megakaryocyte growth and development factor (MGDF) is the major physiological regulator of megakaryocytes and platelet production. The gene for human TPO is located on chromosome 3q27. The mature protein has 332 amino acids. TPO is heavily glycosylated and has an MW of around 70 kDa. TPO is the ligand for the c-MPL receptor, which is present on early hemopoietic progenitor cells, megakaryocytes, and platelets. The m-RNA for TPO is expressed in the liver, kidney, and to a lesser extent in stromal cells of spleen and bone marrow. Signaling via c-MPL involves activation of the JAK[Janus kinase]/STAT and Ras signaling pathways. The serum levels of TPO are high in thrombocytopenic patients and low in normal individuals. TPO stimulates both the proliferation and maturation of cells committed to megakaryocyte

production. Recombinant TPO has been given to patients who were thrombocytopenic following chemotherapy. In these patients, platelets increased with a latency of 8–12 d. The dose of TPO administered was in the range of 0.1–1 µg/kg daily. Platelets produced or stimulated by TPO are functionally normal. The yield of platelets obtained by platelet pheresis from healthy donors treated with TPO is increased two- to threefold, however, in such individuals, the risk of thrombosis with high platelet counts must be considered. According to some studies, TPO is synergistic with other growth factors in mobilizing progenitor cells. Potential clinical applications of recombinant TPO are as prophylaxis for thrombocytopenia following chemotherapy and delayed platelet recovery following autologous transplantation. Recombinant TPO continues to be used in clinical trials for the treatment and prophylaxis of thrombocytopenia. The clinical trials of a pegylated form of TPO were stopped after a subset of patients developed neutralizing antibodies to TPO (with severe thrombocytopenia in a few patients). Currently, a small-molecule agonist of the TPO receptor is undergoing clinical trials for thrombocytopenia.

5.4. Erythropoietin

Erythropoietin (EPO) is produced by kidney cells (in embryonic life also in the liver) and is stimulated by tissue hypoxia. EPO is encoded by a gene on chromosome 7 and has an MW of 1.8 kDa (34–39 kDa in its glycosylated form). It promotes the proliferation and differentiation of erythropoietic cells from progenitor cells. Recombinant EPO is widely used for the treatment of renal anemia (*see* Chapter 5). Subcutaneously injected EPO is also used for other indications such as aplastic anemias, myelodysplastic syndromes, anemias following chemotherapy, and anemias of chronic disease; however, in these indications, the effect of EPO is less predictable. The normal EPO serum levels are between 4 and 26 mU/mL. Recombinant EPO can be expected to improve hematopoiesis if the endogenous EPO level is normal or moderately increased. The recommended dose of recombinant EPO is 100–150 U/kg three times weekly. In patients with renal disease, the recommended dose is lower (50 U/kg three times weekly). Side effects of recombinant EPO include hypertension or hyperviscosity and are mainly observed in patients with renal anemia treated with a high dose of EPO. The improvement in hematocrit may take up to 12 wk after beginning treatment with EPO. EPO is also effective in many cases of HIV-associated anemia (*see* Chapter 17). A new indication for EPO is the anemia observed in preterm infants. At doses of 200 U/kg, most preterm infants improve their hematocrit and many avoid blood transfusions. Another indication of EPO is the treatment and prophylaxis of anemia in Jehova's witnesses who refuse blood transfusions for religious reasons.

Table 1
Currently Known Interleukins and Interferons and Their Characteristics

Interleukins	
Interleukin (IL)-1	Originally described as endogenous pyrogen or lymphocyte-activating factor, it exists in two forms (IL-lα and IL-1β) and is pleiotropic and a mediator of many inflammatory and immunological reactions. IL-1 is secreted by activated monocytes and endothelial cells, cleaved from a precursor peptide (molecular weight [MW] 33 kDa) to a mature protein (MW 17 kDa) by the enzyme IL-1-converting enzyme. IL-1 receptor antagonist occurs as a natural inhibitor of IL-1.
IL-2	Previously known as T-cell growth factor, it has an MW of about 15 kDa and is encoded by a gene on chromosome 4. IL-2 plays a central role in the expansion and activation of antigen-reactive T-lymphocytes. IL-2 has autocrine and paracrine effects on T-cells, but also activates natural killer (NK) cells and other cell types. IL-2 is used in the experimental tumor therapy with lymphocyte-activated killer (LAK) cells and tumor-infiltrating lymphocyte (TIL) cells. LAK cells can be expanded in the presence of 1L-2 and show some activity in patients with melanomas and other cancers. TIL cells have been isolated from tumors and similarly expanded with IL-2.
IL-3	*See* Subheading 5.1.
IL-4	Secreted by activated T-lymphocytes (especially TH2 cells), MW of about 18 kDa, acts both on B- and T-lymphocytes. IL-4 activates quiescent B-lymphocytes and inhibits the action of proinflammatory cytokines on monocytes and macrophages.

(continued)

5.5. Darbepoietin-α

Darbepoietin is a modified form of EPO (five amino acids were modified to permit the attachment of two additional carbohydrate side chains). This modification leads to a more than threefold longer in vivo half-life. The indications for darbepoietin approved by the FDA are anemia of chronic renal insufficiency and anemia secondary to chemotherapy. The effects and side effects of darbepoietin are comparable to EPO. The advantage for patients is less-frequent dosing. The recommended initial dose in patients with chronic kidney failure is 30–50 μg/kg given intravenously or subcutaneously. The target hemoglobin in kidney failure is 11–12 g/dL. In patients who receive chemotherapy, one injection of darbepoietin per cycle was found effective to prevent anemia.

Table 1 (Continued)

Interleukins	
IL-5	Homodimeric protein with an MW of about 50 kDa, secreted by activated T-cells and mast cells, major biological function is to promote the growth and differentiation of eosinophils. Increased serum levels of IL-5 have been found in hypereosinophilic syndromes.
IL-6	Pleiotropic cytokine (MW 26 kDa), produced by activated monocytes, macrophages, endothelial cells, fibroblasts, and some tumor cells. Major stimulators of IL-6 production are other cytokines like tumor necrosis factor (TNF)-α or IL-1, mediator of acute phase and inflammatory reactions, stimulating the growth of differentiated B-cells and the generation of cytotoxic T-cells. IL-6 acts on hematopoietic progenitor cells together with colony-stimulating factors and promotes the growth of myeloid and megakaryopoietic colonies. IL-6 acts as an autocrine growth factor in multiple myeloma and other malignancies.
IL-7	Glycoprotein, with an MW of 25 kDa, stimulates early B- and T-cells, is expressed in the stromal cells of thymus, spleen, bone marrow, and other tissues, and is recognized as a growth factor for mature T-cell lymphomas such as Sezary syndrome.
IL-8	Small molecule secreted by monocytes and stromal cells (chemokine), recruits granulocytes, is involved in the pathogenesis of adult respiratory distress syndrome, and has pyrogenic activities.
IL-9	Pleiotropic cytokine secreted by activated CD4 cells, has an MW of 30–40 kDa. IL-9 acts on T-cells and mast cells, has a synergistic activity with IL-3 on early erythropoietic cells. m-RNA for IL-9 is found in tumor cells of Hodgkin's disease and anaplastic large-cell lymphomas.
IL-l0	Anti-inflammatory properties, MW 18 kDa, produced by activated T-cells, B-cells, and macrophages. IL-10 inhibits the secretion of proinflammatory cytokines like TNF-α, IL-I, IL-6, IL-11, and interferon (IFN)-γ. The apoptosis of B-cells is inhibited in the presence of IL-10.
IL-11	Cloned from stromal cells, is considered as an additional hemopoietic growth factor. IL-11 stimulates the development of megakaryocytes (together with other cytokines), promotes the hematopoietic reconstitution after chemotherapy, and has immunomodulatory effects. IL-11 has an MW of approx 20 kDa. Recombinant IL-11 (oprelvekin) was approved by the Food and Drug Administration for the prevention of severe thrombocyto-

(continued)

Table 1 (Continued)

Interleukins	
	penia and patients with nonmyeloid malignancies after myelosuppressive chemotherapy. The recommended dose of oprelvekin is 50 μg/kg once daily (given subcutaneously).
IL-12	Exists as a heterodimer, has an MW of 75 kDa, is secreted by monocytes or macrophages after stimulation by endotoxin or by activated B-lymphocytes. IL-12 strongly induces the secretion of IFN-γ by T-cells and NK cells and augments the cytotoxicity of NK cells. IL-12 also induces the proliferation of activated T- and NK cells.
IL-13	Structural homology with IL-4 is produced by a subpopulation of activated T-lymphocytes. IL-13 activates B-lymphocytes (e.g., IL-4).
IL-15	Produced by epithelial cells and monocytes, stimulates the proliferation of activated T-lymphocytes. IL-15 has a functional similarity to IL-2 and binds with the β and γ chain of the IL-2 receptor.
IL-16	IL-16 monomer has an MW of 14 kDa, is produced by CD8-positive T-cells, serves as a chemoattractant for CD4-positive lymphocytes, eosinophils, and monocytes. IL-16 binds to the CD4 molecule and is considered to be immunomodulatory and proinflammatory.
IL-17	Produced by activated T-cells, proinflammatory activities. IL-17 induces the production of other cytokines (e.g., IL-6 and IL-8) from stromal cells.
IL-18	Previously described as IFN-γ-inducing factor, related to the IL-1 family. IL-18 is pleiotropic, but generally has proinflammatory activities. Similar to IL-10, IL-18 requires the IL-1β-converting enzyme for cleavage to its active form. Most activities of interleukin-18 are due to a receptor complex that recruits the IL-1 receptor-activating kinase and the consequent translocation of nuclear factor (NF)-κB.
IL-19	Homolog of IL-10, activity at present not well described.
IL-20	Homolog of IL-10, activates keratinocytes.
IL-21	Pleiotropic cytokine, influences proliferation, effector function, and differentiation of B-, T-, NK, and dendritic cells. Has a private receptor (IL-21 receptor) which activates the JAK/signal transducers and activators of transcription pathway upon ligand binding).
IL-22	Induces inflammatory responses.
IL-23	Induces IFN-γ production and proliferation in T-cells.

(continued)

Table 1 (Continued)

Interleukins	
IL-24	Member of IL-10 family, produced by activated monocytes and T-helper cells, can function through receptors or intracellularly as cytotoxic agent.
IL-25	Member of IL-17 family, induces production of IL-4, IL-5, and IL-13.

5.6. Stem Cell Factor

Stem cell factor (SCF) is the ligand for the c-kit proto-oncogene. SCF has an essential role in embryonic development and serves as a growth factor of early hematopoiesis. In vitro, SCF acts synergistically together with G-CSF, EPO, and IL-3 in stimulating hematopoietic colonies. SCF also plays a critical role in mast cell production and function, melanocyte production, germ cell function, and gastrointestinal motility. The gene for human SCF is localized on chromosome 12. When administered to experimental animals, SCF leads to an increase in red blood cells, neutrophils, lymphocytes, eosinophils, and basophils. The receptor for SCF (c-kit) is expressed in acute myelogenous leukemias and certain lymphomas. Human SCF is available in Australia, New Zealand, and Canada as a recombinant protein (ancestim) and was found to increase the mobilization of peripheral blood stem cells in patients who failed a previous mobilization with chemotherapy and/or G-CSF. The recommended dose is 20 μg/kg/d subcutaneously. Because some patients developed anaphylactoid reactions due to mast cell activation, a premedication with inhaled β-mimetics and antihistamines is recommended.

5.7. FLT3 Ligand

FLT3 ligand is a cytokine widely expressed in human tissues. A transmembrane form can be cleaved to generate a soluble form that also has biological activity. The FLT3 receptor is a tyrosine kinase and has a restricted expression (early myeloid and early lymphoid progenitor cells, myeloid leukemias, and certain lymphomas). Based on studies of its expression and function, FLT3-ligand is categorized together with SCF as an early hematopoietic cytokine. Both cytokines require the interaction with other early acting or lineage-specific cytokines. In contrast to SCF, FLT3-ligand does not act on early erythroid cells. A potential clinical application of FLT3-ligand is the expansion of stem cell grafts. FLT3-ligand also stimulates lymphoid progenitors, dendritic cells, and NK cells. Activating mutations of the FLT3 receptor are observed in about 30% of acute myelogenous leukemias (*see* Chapter 9).

5.8. Tumor Necrosis Factor-α

TNF-α is a protein with an MW of 17 kDa, produced mainly by activated monocytes and encoded by a gene on chromosome 6. TNF was originally characterized by its action on certain murine tumors. The physiological relevance of TNF is cell-to-cell interaction and immunoregulation. Lymphotoxin is a related cytokine produced mainly by lymphoid cells. Circulating TNF can be measured in septic shock (endotoxemia) and during acute graft-vs-host reactions. The systemic treatment with TNF in human cancer has showed considerable toxicity and has had only sporadic antitumor effects.

5.9. Fas Ligand

Fas ligand, like TNF, is a type II transmembrane protein with an MW of 38–40 kDa. Fas ligand is produced by activated T- and NK cells, for example, in areas of immune privilege like the testis or the anterior eye chamber. An ectopic expression of fas ligand is found in tumor cells such as colon cancer and melanoma, Fas ligand is cleaved by metalloproteinases and interacts with a receptor present on activated cells and other cell types (Fas, CD95). The physiological function of fas ligand is the transmission of death signals to sensitive cells.

5.10. TNF-Related Apoptosis-Inducing Ligand

TNF-related apoptosis-inducing ligand (TRAIL) is a type II membrane-bound TNF family ligand that is homologous to Fas ligand. TRAIL has the unique property of selectively killing tumor cells and sparing most normal cells. The main function of TRAIL is to induce apoptosis in sensitive cells and activate the transcription factor nuclear factor (NF)-κB. TRAIL has five receptors: DR4 and DR5 transmit death signals, DcR1 and DcR2 act as decoy receptors, and a soluble receptor (osteoprotegerin). The tumor specificity of TRAIL is unclear, but may be related to its intracellular signal transduction.

5.11. Transforming Growth Factor-β

TGF-β is a 14-kDa molecule that is synthesized as a 25–28 kDa homodimeric polypeptide. Three different isoforms of TGF-β are expressed in many tissues, especially in lymphoid cells, monocytes, megakaryocytes, and platelets. The dominant activity of TGF-β is suppression of cell growth, which is illustrated by its ability to inhibit the T-cell response to mitogens. By indirect mechanisms, TGF-β also stimulates some cells, thus promoting angiogenesis and wound healing, for example. TGF-β is expressed in a number of tumor cells including Hodgkin's lymphoma with nodular sclerosis.

5.12. Interleukins

A list of the currently known interleukins (abbreviated as IL-1 through IL-25) and their characteristics are briefly described in Table 1.

5.13. Interferons

IFN-α is widely used for the treatment of hematological diseases (hairy cell leukemia and chronic myelogenous leukemia). IFN-α and IFN-β belong to the group of type I IFNs, whereas IFN-γ is a type II (immune) IFN. Originally, the IFNs were characterized as antiviral substances. However, it was later found that their main activity is immunomodulation. The type I interferons, for example, stimulate the activity of NK cells and modulate the synthesis of immunoglobulins. Moreover, IFN-α has direct effects on tumor cells, such as increasing the expression of cell surface antigens, including class I histocompatibility antigens. This cytokine also has a direct antiproliferative action, possibly through the induction of 2',5'-oligo-adenylate synthetase and inhibits several cell growth-associated proteins. The dose of IFN-α used for the treatment of blood diseases is administered subcutaneously in the range of 3×10^6 U given three times a week to 5×10^6 U given daily. This dose often must be modified, however, as a result of such side effects as fever, chills, myalgias, lethargy, and, less frequently, cardiovascular and metabolic disturbances, interstitial nephritis, confusion, and neuropathies. The pyrogenic side effects subside spontaneously in most cases, and can be controlled with antipyretics. For other side effects, the dose of IFN needs to be altered or the treatment discontinued. About 3–5% of patients develop neutralizing antibodies that render the treatment ineffective. Common hematological side effects of IFN-α are neutropenia and thrombocytopenia.

IFN-β. IFN-β has been found to be less effective than IFN-α against hairy cell leukemia and chronic myelogenous leukemia. A positive response to IFN-β treatment has recently been observed in patients with multiple sclerosis.

IFN-γ. IFN-γ is produced by activated T-lymphocytes. The immunological and antiviral effects of IFN-γ are different from those of the type I IFNs. IFN-γ activates monocytes and macrophages, leads to an increase in the expression of Fc receptors, augments the production of superoxide, and enhances phagocytosis and bactericidal capacity. It increases the cytotoxicity of NK cells and the immunoglobulin synthesis of B-lymphocytes. Like IFN-α, IFN-γ inhibits the growth of some tumor cells in vitro and enhances the cell surface expression of numerous antigens including class I and class II histocompatibility antigens. Clinically, IFN-γ is less active than IFN-α in the treatment of hematological disorders. More

recently, it was shown that children with the granulocyte defect chronic granulomatous disease have fewer infections when treated with subcutaneous IFN-γ.

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http://www.springer.com/978-1-58829-557-6

Modern Hematology
Biology and Clinical Management
Munker, R.; Hiller, E.; Glass, J.; Paquette, R. (Eds.)
2007, XIV, 498 p. 62 illus., 18 illus. in color., Hardcover
ISBN: 978-1-58829-557-6
A product of Humana Press