APLASTIC ANEMIA

Pathophysiology and treatment

Edited by Hubert Schrezenmeier and Andrea Bacigalupo

CAMBRIDGE

more information - www.cambridge.org/0521641012

This page intentionally left blank

APLASTIC ANEMIA

Pathophysiology and treatment

This book takes account of the most recent findings in laboratory research and clinical trials to provide a comprehensive and up-to-date reference on the pathophysiology, epidemiology, diagnosis and treatment of acquired and inherited aplastic anemia.

As well as a comprehensive and detailed overview of the pathophysiology of the disease, the international team of authors covers all aspects of management, including the well established approaches of bone marrow transplantation and immunosuppressive treatment, new approaches such as the use of hematopoietic growth factors and escalated immunosuppression, and controversial issues such as stem cell transplantation. The final section concentrates on the inherited syndrome Fanconi's anemia. Much of the recent work in this area has been coordinated by the European Group for Blood and Marrow Transplantation (EBMT). Included here is an important international consensus document with guidelines on treatment of aplastic anemia which combines the results and views of the EBMT with those of the international experts from America and Japan.

Detailed treatment guidelines are given, making this the definitive resource for hematologists and clinicians from other disciplines involved in the management and supportive care of patients with aplastic anemia. Scientists interested in mechanisms of bone marrow failure will also find this an invaluable reference.

Hubert Schrezenmeier is Professor of Medicine in the Free University of Berlin and Chairman of the EBMT Working Party on Severe Aplastic Anemia. **Andrea Bacigalupo** is Director of the Bone Marrow Transplant Centre, Ospedale San Martino, Genoa, and President of the EBMT.

APLASTIC ANEMIA

Pathophysiology and treatment

Edited by

Hubert Schrezenmeier
Free University of Berlin

and Andrea Bacigalupo
Bone Marrow Transplant Centre, Ospedale San Martino, Genoa

CAMBRIDGE
UNIVERSITY PRESS

PUBLISHED BY THE PRESS SYNDICATE OF THE UNIVERSITY OF CAMBRIDGE
The Pitt Building, Trumpington Street, Cambridge, United Kingdom

CAMBRIDGE UNIVERSITY PRESS
The Edinburgh Building, Cambridge CB2 2RU, UK
40 West 20th Street, New York, NY 10011-4211, USA
477 Williamstown Road, Port Melbourne, VIC 3207, Australia
Ruiz de Alarcón 13, 28014 Madrid, Spain
Dock House, The Waterfront, Cape Town 8001, South Africa

http://www.cambridge.org

First published in printed format 1999

ISBN 0-511-03625-6 eBook (Adobe Reader)
ISBN 0-521-64101-2 hardback

Contents

Contributors

Blanche P. Alter
Division of Pediatric
Hematology/Oncology
Children's Hospital C3.270
University of Texas Medical Branch
Galveston, TX 77555-0361
USA

Andrea Bacigalupo
IInd Division of Hematology
Ospedale San Martino
Viale Benedetto XV
I-16132 Genova
Italy

Manuel Buchwald
Department of Molecular and Medical
Genetics
The Hospital for Sick Children
University of Toronto
Toronto, Ontario
Canada

Bruce M. Camitta
Medical College of Wisconsin
PO Box 26509
8701 Watertown Plank Road
Milwaukee, WI 53226
USA

Madeleine Carreau
Department of Molecular and Medical
Genetics
The Hospital for Sick Children
University of Toronto
Toronto, Ontario
Canada

H. Joachim Deeg
Clinical Research Division
Fred Hutchinson Cancer Research
Center
1124 Columbia Street
Seattle, WA 98104
USA

Frances M. Gibson
Department of Haematology
St George's Hospital Medical School
Cranmer Terrace
London SW17 0RE
UK

Eliane Gluckman
Service de Greffe de Moelle Osseuse
Hôpital Saint-Louis
1 Avenue Claude Vellefaux
F-75475 Paris, Cedex 10
France

E. C. Gordon-Smith
Department of Haematology
St George's Hospital Medical School
Cranmer Terrace
London SW17 0RE
UK

Philippe Guardiola
Service de Greffe de Moelle Osseuse
Hôpital Saint-Louis
1 Avenue Claude Vellefaux
F-75475 Paris, Cedex 10
France

Hermann Heimpel
University of Ulm
Robert-Koch-Str aße8
D-89081 Ulm
Germany

Jill M. Hows
Division of Transplant Sciences
Southmead Health Services
Bristol
BS10 5NB
UK

Seiji Kojima
Department of Developmental Paediatrics
Nagoya University School of Medicine
Nagoya 453
Japan

Johnson M. Liu
Hematology Branch
NHLBI, NIH
10/ACRF/7C103
Bethesda, MD 20892
USA

Per Ljungman
Department of Hematology
Karolinska Institute
Huddinge University Hospital
SE-14186 Huddinge
Sweden

Anna Locasciulli
Division of Pediatric Hematology
Ospedale San Gerardo
Via Donizetti 106
I-20052 Monza
Italy

Pedro Marín-Fernandez
Postgraduate School of Hematology (EHFC)
Ferreras Valenti Hospital Clinic
C/Villarroel 170
E-08036 Barcelona
Spain

Judith C. W. Marsh
Department of Haematology
St George's Hospital Medical School
Cranmer Terrace
London SW17 0RE
UK

Jean-Yves Mary
Centre de Bioinformatique
INSERM U444
Université Paris VII
Paris
France

Shaun R. McCann
Department of Haematology
St James's Hospital
Dublin 8
Ireland

Shinji Nakao
Third Department of Medicine
Kanazawa University School of Medicine
13-1 Takaramachi, Kanazawa
Ishikawa 920
Japan

Jakob R. Passweg
Department Innere Medizin
Kantonsspital Basel
Petersgraben 4
CH-4031 Basel
Switzerland

N. J. Philpott
Department of Haematology
St George's Hospital Medical School
Cranmer Terrace
London SW17 0RE
UK

Aruna Raghavachar
Dept of Internal Medicine III
University of Ulm
Robert-Koch-Straße8
D-89081 Ulm
Germany

Hubert Schrezenmeier
Benjamin Franklin University Hospital
Medical Clinic III
Free University of Berlin
Hindenburgdamm 30
D-12200 Berlin
Germany

Gérard Socié
Bone Marrow Transplantation Unit
Hôpital Saint-Louis
1 Avenue Claude Vellefaux
75475 Paris Cedex 10
France

Judith Veum Stone
Statistical Center
Medical College of Wisconsin
PO Box 26509, 8701 Watertown Plank Road
Milwaukee, WI 53226
USA

Rainer Storb
Clinical Research Division
Fred Hutchinson Cancer Research Center
1124 Columbia Street
Seattle, WA 98104
USA

Nydia G. Testa
Department of Experimental Haematology
Paterson Institute for Cancer Research
Wilmslow Road
Manchester M20 9BX
UK

André Tichelli
Hematologie
Kantonsspital Basel
Petersgraben 4-8
CH-4031 Basel
Switzerland

Preface

Recently important progress has been made to improve our understanding of the pathophysiology of aplastic anemia and the clinical management of this rare disease.

For a long time the pathophysiology of aplastic anemia remained a mystery, until a series of new studies provided new insight into this matter. The new information includes demonstration of a defect in bone marrow in those with aplastic anemia at the level of long-term culture-initiating cells; further evidence for the pathophysiological relevance of inhibitory cytokines; the assessment of cytokine action in vitro on bone marrow in aplastic anemia, and the analysis of cytokine expression in aplastic anemia; new results on autoreactive T-cells in aplastic anemia; evidence of roles for Fas-antigen and apoptosis in the pathophysiology of aplastic anemia; the elucidation of the relationship between aplastic anemia and paroxysmal nocturnal hemoglobinuria at the molecular level; and new data on the issue of clonality in bone marrow failure.

The main treatment options for aplastic anemia are bone marrow transplantation and immunosuppressive treatment. Progress in bone marrow transplantation for aplastic anemia includes new conditioning regimens and increasing the number of transplants in aplastic anemia from alternative donors.

During the 1990s a series of clinical studies on treatment with immunosuppression and growth factors were performed. These studies helped to improve standard immunosuppressive treatment. There are, however, still many questions on the place of hemopoietic growth factors in the treatment of aplastic anemia.

Efforts were also focused to analyze late effects of all treatment modalities.

Thus, there is a substantial, recently established body of information on the pathophysiology and treatment of aplastic anemia. This book tries to summarize the established knowledge and the most recent progress in the subject of aplastic anemia.

The idea for this book was generated within the Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT). However, we tried to avoid restriction to an 'EBMT-view' of the disease and we were fortunate in persuading distinguished experts from many countries to contribute to this volume.

The editors would like to express their appreciation for support of the work of members of the EBMT Aplastic Anemia Working Party by a grant from the European Commission (Biomed-2 programme, contract no. BMH4-CT96-1031).

We are sincerely grateful to all contributors for their excellent work; our thanks to all of them. We hope that this book will be a source of helpful, up-to-date information for students, clinicians, scientists and patients.

Hubert Schrezenmeier
Andrea Bacigalupo

Part I

Pathophysiology of acquired aplastic anemia

1

Stem cell defect in aplastic anemia

Judith C.W. Marsh

St George's Hospital, London

and

Nydia G. Testa

University of Manchester

Abbreviations

AA	Aplastic anemia
ADA	Adenosine deaminase
BFU-E	Burst-forming unit, erythroid
BL-CFC	Blast-colony forming cells
CAFC	Cobblestone area forming cells
CFU-E	Colony-forming unit, erythroid
CFU-GM	colony-forming unit, granulocyte/macrophage
CSF	Colony-stimulating factor
5-FU	5-Fluorouracil
G-CSF	Granulocyte colony-stimulating factor
GM-CFC	Granulocyte/macrophage colony-forming cells
HPP-CFC	High proliferative potential-colony forming cells
IL-1	Interleukin-1
IFN-γ	Interferon-γ
LTBMC	Long-term bone-marrow culture
LTC-IC	Long-term culture-initiating cell
LTRC	Long-term repopulating cells
M-CSF	Macrophage colony-stimulating factor
MIP1α	Macrophage-inflammatory protein α
Mix-CFC/CFU-Mix	Mixed lineage multipotential colony-forming cells
NOD/SCID	Nonobese diabetic, severe-combined-immunodeficient mice
SCF	Stem cell factor
TGFβ	Transforming growth factor β
TNF-α	Tumor necrosis factor α

Normal stem cells

Definition

The need to continuously replace mature cells in the blood requires the production of about 10^{11} new cells daily in a normal adult, and even more in response to hemopoietic stress. It is known that all these cells are derived from a common ancestor population, the pluripotential stem cells (Lajtha, 1983). The usually accepted definition of stem cells is based on three characteristics: first, their marked capacity for proliferation, as just illustrated, and, second, their potential to undergo differentiation to produce all the lymphohemopoietic mature cell types (Metcalf, 1988). Third, and classically, stem cells are also defined by their reported capacity for self-renewal, i.e., the capacity to generate new stem cells, with the implication that they are able to regenerate their own population (Lajtha, 1983; Metcalf, 1988). As we will discuss later, it is mainly this latter concept that has to be discussed in the context of aplastic anemia (AA).

Regulation

Stem cells comprise only a small minority, between 0.01 and 0.05%, of the total cells found in bone marrow. At least 95% of the hemopoietic cells fall into morphologically recognizable types. The remaining, with nonspecific morphological features, require phenotypic and functional characterization. They encompass not only the stem cells but also their immediate progeny, the progenitor cells which were first characterized by their ability to develop in vitro in response to the colony-stimulating factors (CSF) (Metcalf, 1988). As the hemopoietic tissue is a continuum of differentiating and proliferating cells, the boundaries between different primitive cell populations are ill-defined. However, it is generally agreed that commitment, i.e., a decision to enter a particular differentiation lineage (therefore restricting the multipotentiality of the stem cells), distinguishes between stem and progenitor cell populations. Such commitment appears to be irreversible. For example, macrophage progenitors which are genetically manipulated to present the erythropoietin receptors still develop into macrophages following stimulation with erythropoietin (McArthur et al., 1995). In the converse experiment, erythroid progenitors induced to express the receptor for macrophage colony-stimulating factor (M-CSF) develop into erythroid cells in response to this cytokine (McArthur et al., 1994). In contrast, little is known about the commitment process itself and, at present, the argument rages whether such an event is dictated internally by the cell-driven program, or is the response to external, i.e., environmental, stimuli (Jimenez et al., 1992; Ogawa, 1993). These concepts are important to the under-

standing of AA, since stem cells, which must supply mature functional cells for a lifetime, must be protected from the competing demands for mature cells in response to physiological or pathological needs. The rapid response of the hemopoietic tissue is met by the more mature, differentiation-restricted progenitors; for example, BFU-E cells (burst-forming unit; erythroid) in response to hypoxia or blood loss, or granulocyte/macrophage colony-forming cells (GM-CFC) in response to infection. These, and equivalent cell populations in the other lineages, are largely controlled by growth factors. However, the steps that *generate* these progenitors from stem cells are, at present, unknown. In the context of AA, it is interesting to speculate whether, in some cases, the 'protection' mechanisms that may act to protect the stem cell population from exhaustion are defective. As stem cells are lodged within bone marrow stroma, it is generally assumed that those stromal cells produce and may present a membrane-bound form, or release a large number of regulatory cytokines, including stimulatory molecules such as interleukin-1 (IL-1), M-CSF, GM-CSF, G-CSF, IL-6 and stem cell factor (SCF), as well as inhibitory cytokines such as transforming growth factor β (TGFβ) and macrophage-inflammatory protein α (MIP1α, for review see Lord et al., 1997). However, whether they have a role in stem cell differentiation is not known. Some do have a role, at least in vitro, in their survival and proliferation (Fairbairn et al., 1993). It may very well be that regulatory factors crucial for the commitment to differentiation are still unknown; such factors may have more in common with those which regulate embryonic and fetal development than with the 20–30 cytokines known to regulate the proliferation, maturation and function of the committed hemopoietic progenitors and their developing progeny (Lord et al., 1997).

The experimental study of primitive hemopoietic cells

While assays for colony-forming cells detect mainly progenitor cells, transplantation experiments define stem cells by their function, i.e., their capacity to repopulate permanently the hemopoietic tissue (Table 1.1). The long-term repopulating cells (LTRC) can, at present, be assayed only in experimental systems.

Currently, the most primitive human cell that can be assayed in vitro is the long-term culture-initiating cell (LTC-IC) (Table 1.1). This cell has certain stem cell characteristics, but it is not yet clear how it is related to the human stem cell. However, Ploemacher (1994) showed that murine LTC-IC (assessed as cobblestone area forming cells or CAFC) were able to repopulate irradiated mice and could, therefore, be regarded as equivalent to the mouse repopulating cell. A number of animal models have been developed for transplantation studies. Sublethally irradiated, severe-combined-immunodeficient, nonobese diabetic (NOD/SCID) mice were used to test the engraftment and repopulating potential

Table 1.1. Assays for primitive stem and progenitor cells

Cells	Assay	Incidence in bone marrow	References*
Long-term repopulating cells (LTRC)	Reconstitution of hemopoietic tissue	$1/(5\times10^4$–$10^5)$	Lord et al., 1997; Ploemacher, 1994
Long-term culture initiating cells (LTC-IC)	Generation of progenitor cells (CFC after 5–8 weeks of culture)	$1/(10^4$ to $2\times10^4)$	Lord et al., 1997; Ploemacher, 1994; Testa et al., 1996
Also called cobblestone area forming cells (CAFC)	Generation of a cobblestone area of cell proliferation		
Multipotential colony-forming cells (CFC)** HPP-CFC BL-CFC Mix-CFC	Colony formation in vitro	$1/(5\times10^4$–$10^5)$	Lord et al., 1997; Metcalf, 1988
Bipotential CFC	Colony formation in vitro	1–$2/10^3$ according to lineage	Lord et al., 1997; Metcalf, 1988

Notes:

* Mostly reviews are quoted.

** Definitions in the text and beginning of chapter.

of putative human stem-cell populations. However, limiting dilution repopulation assays indicate that the frequency of a NOD/SCID mouse repopulating cell is 1 in 10^6 cord blood mononuclear cells, whereas 1 in 3×10^3 to 10^4 mononuclear cells was an LTC-IC (Pettengel et al., 1994). Clearly, the human repopulating cells, as assessed in the NOD/SCID model, appear to be more primitive than the human LTC-IC. Furthermore, a gene-transfer study using a retroviral adenosine deaminase (ADA) vector showed that 30–40% of colony-forming cells and LTC-IC could be transduced with the ADA vector, but, when the cells were transplanted into NOD/SCID mice, none of the colony-forming cells generated were positive for ADA. Although high numbers of colony-forming and mature cells were obtained, it seems that the transfected cells contributed little to the graft, and the cells responsible for repopulation were not transfected. This further indicates that the repopulating cell may be more primitive than the LTC-IC. On the other hand, potentially lethally irradiated mice can be rescued from hemopoietic death by 5×10^4 to 10^5 bone marrow cells (Lord et al., 1997). It is not clear whether the larger numbers of human cells required to rescue the irradiated NOD/SCID mice mean that there are fewer stem cells in humans than in mice. However, there are problems with the maturation of human cells in those mice (Larochell et al., 1996), which suggests that the relatively low incidence of LTRCs in this system may be an assay-driven paradox. The clonogeneic in vitro assays detect mainly the progenitor cells, which are more mature than stem cells. However, because of the continuous spectrum of proliferation and differentiation in the hemopoietic tissue, some of the clonogeneic assays may partially overlap with the stem cell compartment. The blast colony assay (BL-CFC) and the high proliferative potential colony assay (HPP-CFC) are within this category.

Selection of primitive cells by phenotype

It is possible to separate the most primitive cells from their close progeny of progenitor cells. The former have distinct cell membrane markers (Table 1.2) and are also characterized by low metabolic activity. This latter feature allows primitive cells to be isolated by negative selection, using dyes such as rhodamine-123, which concentrates in active mitochondria, or nucleic acid dyes like Hoechst 33342 (Ratajczak and Gerwitz, 1995; Spangrude, 1994).

One of the most useful membrane markers for the selection of primitive cells has been the CD34 antigen, and this feature has been exploited in a number of different positive cell-selection procedures (de Wynter et al., 1995). However, the cells that are CD34+ comprise a wide population, encompassing stem cells, progenitor cells and more differentiated hemopoietic cells. In fact, only 0.1–1% of the CD34+ cells have the most primitive phenotype, while about 10–30% are progenitor cells, and the rest are more differentiated cells (Table 1.3).

Table 1.2. Phenotypic markers of primitive hemopoietic cells

Stem cells	Progenitor cells
CD34+	CD34+
CD38−	CD38+
CD33−	CD33+
Lineage−	Lineage+
HLA-DR− or weakly +	HLA-DR− or weakly +
CD71−	CD71+
Thy 1 *low*	Thy 1+
CD45RA *low*	CD45RA+
c-*kit*+	c-kit *low* or −

Note:
Reviewed in de Wynter et al., 1995; Lord et al., 1997; Spangrude, 1994; Testa et al., 1996.

Table 1.3. Percentage of colony-forming cells (CFC) in the different CD34+ subpopulations expressing stem and progenitor cell phenotype

Phenotype	Percentage of cells	Percentage of CFC
CD34+38+DR+	90	31
CD34+38+DR−	4	N.D.
CD34+38−DR+	6	1.0
CD34+38−DR−	0.3	0.2

Note:
Data calculated from Wynn et al., 1998; N.D. = not determined.

How many stem cells get to express themselves?

Recently, a study demonstrated that one injected cell with a 'stem cell' phenotype is able to reconstitute long-term hemopoiesis in an irradiated mouse (Osawa et al., 1996). The proportion of mice injected with single cells that were reconstituted agrees with the expected proportion (about 20%) of cells seeding in the bone marrow (Testa et al., 1972). Other transplantation studies with marked murine cells have also demonstrated that monoclonal or oligoclonal hemopoiesis may be observed for long periods of time (Capel et al., 1988; Keller and Snodgrass, 1990). Only limited data are available in larger mammals; in experiments with cats, small numbers of syngeneic stem cells are able to maintain hemopoiesis (Abkowitz et al., 1995).

In humans, normal hemopoiesis is polyclonal, and polyclonal hemopoiesis is also usually observed following allogeneic transplantation. Nevertheless, there are anecdotal reports of oligo- or monoclonal hemopoiesis after allogeneic transplantation. This was observed in two out of 12 cases by examining X-chromosome-linked polymorphisms, one of them limited to myeloid cells and the other also comprising lymphoid cells (Turhan et al., 1989). Unfortunately, these observations were made days or weeks after transplant, and the long-term features of hemopoiesis in those patients are not known. However, oligoclonal hemopoiesis, as determined by cytogenetic marks on atomic bomb survivors, may be observed for several years (Amenomori et al., 1988). In one patient, a single identifiable clone provided about 10% of all the lymphohemopoietic cells for an observation period of 10 years, in the absence of any detectable sign of abnormal hemopoiesis (Kusunoki et al., 1995).

Recent studies of normal subjects showed that about 30% of females aged 70 years or older had oligoclonal hemopoiesis in the myeloid, but not the lymphoid lineages (Champion et al., 1997; Gale et al., 1997). It is not clear whether this is caused by altered regulation of cell production or a limited supply of stem cells in the aged. These data, taken together, suggest that only a few stem cells may, under normal steady state, be needed to maintain normal hemopoiesis. They also confirm the early concept that the stem cell population is normally quiescent, and that only a fraction of their vast reserve population needs to express itself, differentiating and giving rise to progeny. In the context of AA, these data also suggest that a mere reduction of stem cell numbers may not be sufficient to cause this syndrome. It is also important to consider how many of the available stem cells are likely to proliferate in AA.

Progressive telomere shortening of CD34+ occurs with age (Vaziri et al., 1994), and we have shown, in paired studies of donors and recipients of allogeneic transplantation, that the telomere length of the recipient's blood cells is significantly shorter than that of their donors. Such shortening is equivalent to that observed during 15 years of normal aging and, in the worst cases, is equivalent to 40 years (Wynn et al., 1998).

Although we do not yet know the molecular mechanisms of this phenomenon, we attribute the accelerated telomere shortening to proliferation stress. If stem cells age, do they conserve their capacity for self-reproduction after the hemopoietic system has reached its adult size? Cultures of human hemopoietic cells have achieved marked expansion of CFC and of LTC-IC (reviewed in Testa et al., 1999), but it is more problematic to assess whether stem cells have increased in number, as assessed by an increase in their capacity to regenerate hemopoiesis. While a primitive phenotype may be conserved, the repopulation capacity may be decreased (Albella et al., 1997). Because of this, it is not known whether the numbers of cells needed for transplantation will be the same when using freshly harvested cells or cells expanded in vitro. Experiments on mice

indicate that 6-fold to 50-fold more in-vitro-generated GM-CFC are required to achieve an equivalent number of leukocytes in the blood (Albella et al., 1997). Therefore, it is doubtful that significant expansion of the stem cell population has been achieved. This may not be surprising since the stimulatory cytokines used in those experiments are those known to act on the progenitor cell populations.

Extensive data have also been obtained from experimental systems and patients. Such data indicate that, following serious cytotoxic injury, the stem cell population recovers to a lesser extent than more mature populations, and remains at markedly subnormal levels for the rest of the experimental animal's life, and for several years at least in patients (reviewed in Testa et al., 1996). Progenitor and maturing cell populations have evolved in response to selective pressures that stimulate hemopoiesis, such as infection and blood loss. In contrast, the use of irradiation and the cytotoxic drugs that kill stem cells were developed recently, in the twentieth century; therefore, it is not surprising that they have not developed mechanisms to normalize their numbers after injury. Fortunately, as discussed above, first, their normal numbers far exceed those needed for a normal life span, and second, an adequate output of mature cells may be reached even with a severely restricted stem cell compartment. However, it is apparent that the concept that hemopoietic stem cells in the adult have the capacity to self-reproduce has to be revised. Perhaps it is more realistic to think that while stem cells are characterized by a very extensive proliferation capacity, each cell division results in some stem cell aging. Thus, while operationally the daughter cells may still be defined as stem cells, they are not identical to the parent cell.

Aplastic anemia stem cells

Functional assessment of AA hemopoietic progenitor cells

Early work from the 1970s, with clonogeneic cultures using unpurified bone marrow mononuclear cell preparations and various conditioned media as a source of colony-stimulating activity, demonstrated a reduction or absence of late and early colonies (CFU-GM, CFU-E, BFU-E and CFU-Mix) in patients with AA (Barrett et al., 1979; Hara et al., 1980; Kern et al., 1977). Although variation in colony numbers was seen between individual patients, there was a uniform lack of correlation with disease activity in terms of peripheral blood neutrophil count or marrow granulocytic precursors. Numbers of peripheral blood colonies were at least 10-fold less than bone marrow colonies and more often undetectable. More recent studies using purified (CD34+) hemopoietic cells and recombinant hemopoietic growth factors in clonogeneic culture confirm the reduced numbers

of all marrow progenitor cells (Maciejewski et al., 1994; Marsh et al., 1991; Scopes et al., 1996).

The long-term bone-marrow culture (LTBMC) system has been used by several groups to (1) evaluate the earlier stages of hemopoiesis and (2) assess the ability to form a normal stromal layer, the in vitro representation of the marrow microenvironment. All studies of AA patients have demonstrated a marked defect in hemopoiesis, as manifest by a severe reduction in, or cessation of, the generation of hemopoietic progenitor cells within the system (Bacigalupo et al., 1992; Gibson and Gordon–Smith, 1990; Holmberg et al., 1994; Marsh, 1996; Marsh et al., 1990). A similar pattern is seen in untreated patients, whether with severe or nonsevere disease, and treated patients who have responded hematologically to immunosuppressive therapy (Marsh et al., 1990).

The formation of the stromal layer is normal in most patients with AA (Gibson and Gordon–Smith, 1990; Marsh et al., 1991), although one study reported a lack of stromal confluency in almost half the patients, and that this was associated with a longer duration of disease (Holmberg et al., 1994). Using a different short-term culture system, Nissen and colleagues (1995) reported impairment of stroma formation at 2 weeks but most became confluent at the standard long-term culture time. In contrast, some AA patients form a confluent layer more rapidly than normal (Marsh et al., 1990).

The defect in hemopoiesis seen in LTBMC may reflect either a failing in the stem cell compartment with a deficiency of primitive cells with marrow-repopulating ability, or a dysfunctional microenvironment. Cross-over LTBMC experiments allow separate examination of these two components. Using AA marrow adherent-cell-depleted mononuclear cells, one group demonstrated defective generation of CFU-GM when the cells were inoculated onto normal irradiated LTBMC stromal layers (Marsh et al., 1990). In contrast, normal stromal function in AA patients was demonstrated by normal numbers of CFU-GM generated from normal marrow mononuclear cells when inoculated onto irradiated stromal layers from AA patients, except in one patient in whom a defective stroma was demonstrated. A second group showed similar results, assessing BL-CFC generation on irradiated stromal layers in AA (Novotski and Jacobs, 1995). Furthermore, a similar pattern was seen using purified CD34+ cells as the inoculum, in that the stroma in AA patients supported generation of normal CFU-GM (Marsh et al., 1991) or BL-CFC (Novotski and Jacobs, 1995) from normal marrow CD34+ cells, and purified AA CD34+ cells failed to generate normal numbers of CFU-GM on normal stromas. Hotta and co-workers (1985) had previously demonstrated abnormal stromal function in three out of nine AA patients, although their stem cell function was not examined.

The results of these cross-over experiments indicate a deficiency or defect in primitive cells with marrow-repopulating ability, which in normals had previously been shown to exhibit the CD34+, CD33– phenotype (Andrews et al.,

1989) and within which population LTC-IC are found. Although not all patients form a confluent stroma, in those patients in whom stromal function has been evaluated, in terms of their ability to support the generation of hemopoietic progenitors, the majority function normally. A reported isolated deficiency of a growth factor or increased expression of an inhibitory cytokine (Holmberg et al., 1994) appears not to affect the physiological function of the stroma, as assessed by the long-term marrow-culture system.

Phenotypic quantitation of AA hemopoietic (CD34+) cells

The percentage of bone marrow CD34+ cells is significantly reduced in AA patients compared with normal steady-state bone marrow, with median values of around 0.5%, but with an wide range seen from zero to values falling within the normal range (Maciejewski et al., 1994; Marsh et al., 1991; Scopes et al., 1994). Analysis of the CD34+ subpopulation reveals a significant reduction in the immature CD34+,33– cells, as well as the more mature CD34+,33+ cells (Scopes et al., 1994). A lack of correlation between these compartments and disease severity was reported by one group. Although a second group reported significantly higher percentages of CD34+ and CD33+ cells in patients with recovered AA, almost half the patients had persistently reduced values (Maciejewski et al., 1994). In other words, extreme variability of results was seen among patients who had recovered hematologically after immunosuppressive therapy. It should be remembered that the CD34+ compartment comprises a very heterogeneous collection of cell types in terms of their stage of differentiation, the majority of which comprise the more lineage-restricted progenitors, with the more primitive progenitors comprising only a very small proportion of the CD34+ cells. It appears that, in AA, the CD34+ population contains a much smaller proportion of very primitive cells, with a relative over-representation of more mature progenitors.

AA CD34+ hemopoietic cells have also been shown to be dysfunctional (Scopes et al., 1996). Although marrow mononuclear cells from AA patients consistently produce lower numbers of colonies compared with normal, when the reduced numbers of CD34+ cells in AA bone marrow are considered there is no significant difference in clonogeneic potential. However, when purified AA CD34+ cells cease to be influenced by accessory cells, their clonogeneic potential is significantly reduced, indicating defective function. From the same study, the effects of various hemopoietic growth factors in isolation or in combination on the clonogeneic potential of AA marrow cells was investigated. It was shown that the addition of granulocyte colony-stimulating factor (G-CSF) in vitro was able to correct the dysfunction of AA CD34+ cells to normal in terms of their clonogeneic potential. Thus, in AA there appears to be both a deficiency and a dysfunctionality of marrow CD34+ cells.

Assessment of the long-term marrow-repopulating ability of AA hemopoietic cells

As discussed earlier, the LTC-IC and CAFC assays represent modifications to the LTBMC system to permit quantitation of these primitive hemopoietic cells. Maciejewski and colleagues (1996) demonstrated, by limiting dilution analysis, reduced clonogeneic potential of LTC-IC in two patients; however, for other AA patients examined, limiting dilution analysis was not possible because of low cell numbers. Instead, results of LTC-IC frequency were extrapolated from week-5 clonogeneic cells from bulk cultures and the numbers divided by the average proliferative potential of single AA LTC-IC, based on the small number of formal limiting dilution assays. Using this methodology, the frequency of LTC-IC was reduced compared with normal controls (AA patients had 0.024 colonies/10^5 mononuclear cells compared with 7.8 for normal controls). Furthermore, LTC-IC remained subnormal in those cases, despite achieving normal or near-normal blood counts. LTC-IC were also qualitatively abnormal, demonstrating a markedly reduced clonogeneic potential. Schrezenmeier and colleagues (1996) have also measured the frequency of LTC-IC but used the CAFC as the endpoint for scoring LTC-IC at week 5 instead of the generation of colony-forming cells. They demonstrated a reduction in CAFC in AA patients (mean frequency of CAFC was 6.6/10^5 mononuclear cells (mnc) compared with 84.4 for normal controls). The frequency of LTC-IC is notably higher than reported by Maciejewski et al. (1996), raising questions as to whether the two assay systems are exactly comparable, and whether the CAFC assay detects a somewhat more mature progenitor cell that the LTC-IC (Weaver et al., 1997). In summary, these studies indicate a deficiency in LTC-IC in AA patients, which would account for the deficient marrow-repopulating ability seen in LTBMC.

Podesta and colleagues (1998) have compared the frequency of late hemopoietic progenitors and LTC-IC in AA patients after immunosuppressive therapy with that in AA patients who have undergone successful allogeneic bone marrow transplant (BMT), over a follow-up period of up to 20 years. Although all patients had achieved normal blood counts, bone marrow cellularity and numbers of CFU-GM, BFU-E and CFU-Mix remained subnormal, but there was an even more striking reduction in LTC-IC, equally in transplanted patients and those who had received immunosuppressive therapy (see Figure 1.1). The pattern of recovery of CFU-GM between the two groups was different, with a more rapid normalization of CFU-GM in transplanted patients over a period of 2 years. In contrast, patients treated with immunosuppressive therapy displayed a more prolonged pattern of recovery of CFU-GM over 5–6 years, which may reflect an ongoing process of suppression of hemopoiesis among these patients (see Figure 1.1). From these results, it appears that even a markedly reduced stem cell reservoir (as assessed by LTC-IC frequency) is able to maintain steady-state hemopoiesis, although this

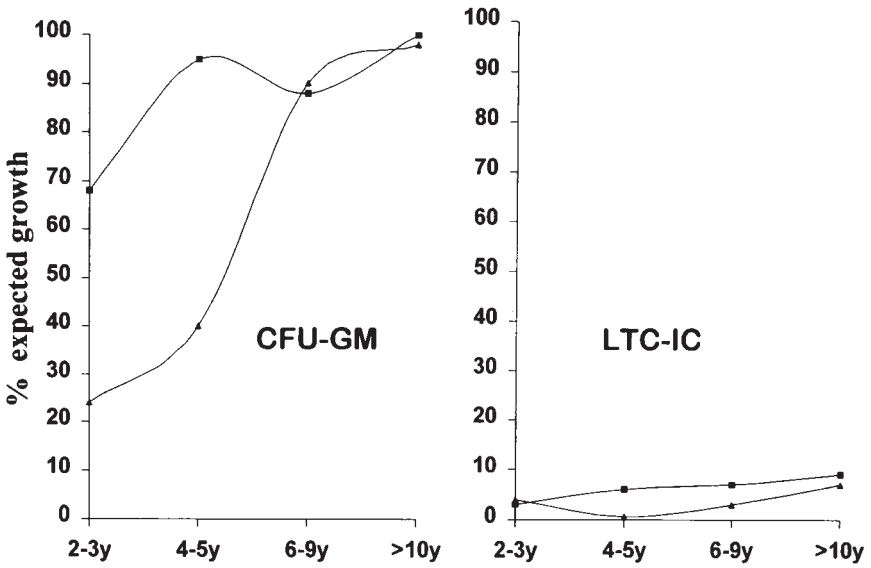

Figure 1.1. Pattern of growth of mixed myeloid cultures (colony-forming unit granulocyte/macrophage or CFU-GM) and long-term culture-initiating cells (LTC-IC) over time following treatment with immunosuppressive therapy (▲) and bone marrow transplantation (■). Numbers are expressed as percentage of expected growth; 100% refers to a median normal of $58/10^5$ mononuclear cells (mnc) for CFU-GM and $34/10^6$ mnc for LTC-IC; y = years from treatment (reproduced from Podesta et al., 1998, with permission).

may not be maintained under conditions of hemopoietic stress. In terms of the quality of the LTC-IC, in the transplanted patients LTC-IC generated normal numbers of colony-forming cells at week 5. In contrast, the proliferative potential of LTC-IC was reduced in patients treated with immunosuppressive therapy, compared with normal controls. This would seem to indicate a qualitative abnormality in stem cells derived from patients who recover autologous hemopoiesis after immunosuppressive therapy compared with the normal quality of stem cells (LTC-IC) grown from AA patients receiving an allogeneic stem cell transplant. Persistence of this abnormality may be one explanation for the risk of relapse of AA or later clonal evolution. An alternative explanation for these results is that most stem cells (and LTC-IC) in AA are unable to enter the cell cycle and proliferate normally. This may be compensated for by increased replicative pressure on the more mature hemopoietic progenitor cells (see 'Analysis of telomeric DNA length in AA', p. 16). From a practical viewpoint, the altered cell cycling status of AA stem cells would impact on an attempt to quantitate LTC-IC and make direct comparison of LTC-IC frequency with that of normal controls difficult. Hence LTC-IC assays may not be suitable for the quantitation of very primitive hemopoietic cells in AA.

Very little is known about the kinetics of stem cell proliferation in AA. Maciejewski and colleagues (1994) examined the expression of c-*kit* on AA CD34+ cells, on the basis that in normal marrow CD34+ c-*kit*+ cells contain the highest proportion of cycling cells. Cell cycle analysis was not performed on AA CD34+ cells, but they showed that the percentage of c-*kit*+ cells among the CD34+ cell population was reduced, suggesting that in AA fewer CD34+ progenitors are

cycling. Preliminary work by Gibson and colleagues (1996) has demonstrated reduced regeneration of progenitors from 5-fluorouracil-treated (5-FU-treated) AA bone marrow cells inoculated onto irradiated LTBMC stromal layers compared with normal 5-FU-treated cells, and that colonies were produced for only 2–4 weeks. This suggests defective or deficient numbers of primitive noncycling stem cells in AA, and also that the finding of reduced or absent LTC-IC in AA may also reflect abnormal proliferation and differentiation kinetics of the stem cells.

Mobilizing potential of AA progenitor cells

It is well established that primitive hemopoietic progenitor cells (including true stem cells from long-term follow-up of allogeneic peripheral-blood stem-cell transplants) can be mobilized from the bone marrow of normal donors using G-CSF (To et al., 1997). However, it may be possible to mobilize residual stem cells from AA patients. Collection and cryopreservation of mobilized stem cells may allow the subsequent use of intensive immunosuppression followed by reinfusion of the stem cells. One group has attempted to collect mobilized blood progenitor cells in AA patients following treatment with antilymphocyte globulin and cyclosporin and 3 months of daily G-CSF (Bacigalupo et al., 1993). The median number of CD34+ cells collected was 1.8×10^6/kg (range 0.27–3.8) and median CFU-GM 3.9×10^4/kg (range 0–39). Colony growth was only obtained on leukaphereses performed between days +33 and +77. There was marked patient variability in terms of mobilizing ability, but in some cases sufficient CD34+ cells were obtained for potential autologous transplantation. It is not known, however, whether any LTC-IC can be isolated using this procedure, and, so far, we are not aware of any report using this approach to treat AA patients.

Apoptotic properties of AA CD34+ cells

It has recently been demonstrated that AA CD34+ marrow cells are more apoptotic than normal CD34+ marrow cells. In addition, there appears to be a correlation between the percentage that is apoptotic and disease severity, and also between the percentage of CD34+ cells present (Philpott et al., 1995). Increased apoptosis may be an important contributory factor to the stem cell defect in AA. Maciejewski and colleagues (1995*a*) had shown that AA CD34+ cells show increased expression of Fas-antigen and that tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) upregulate the expression of Fas-antigen on normal CD34+ cells (Maciejewski et al., 1995*b*). Whether the Fas system is involved in apoptosis in AA remains to be determined. This topic is discussed in detail in Chapter 4. The ability of hemopoietic growth factors such as G-CSF to suppress apoptosis may be an important factor in the effect of G-CSF in vitro and in vivo in AA patients.

Analysis of telomeric DNA length in AA

As discussed earlier, normal hemopoietic stem cells demonstrate progressive telomere shortening with age. A recent study of patients with AA has shown significantly shorter mean telomere length in both granulocytic and mononuclear cell fractions compared with age-matched controls, suggesting some loss at the level of the hemopoietic stem cell (Ball et al., 1998). The degree of telomere loss was proportional to disease duration, and amounted to a loss of 216 base pairs (bp) per year in addition to the normal age-related loss of 36 bp/year. In those patients who had achieved normal blood counts after treatment, the rate of telomere loss had stabilized. It may be that the remaining hemopoietic progenitor cells need to undergo a greater number of cell divisions in order to generate sufficient mature blood cells. This may reflect stem cell loss caused by an increase in apoptosis of stem cells and primitive progenitor cells, or direct immune destruction of these cells. An increase in the replicative capacity of hemopoietic progenitor cells may account for the increased telomere loss in AA patients.

Conclusion

It is now apparent that only a few stem cells are required to maintain normal steady-state hemopoiesis. Normal stem cells exhibit a progressive shortening of telomeric DNA with age, so their self-replicative capacity is not preserved with time. Furthermore, following injury to the stem cells from chemotherapy, for example, the stem cell reservoir does not recover to normal in contrast to the more mature progenitor cells. For these reasons, the classic concept of the hemopoietic stem cell with unlimited self-renewal capacity has been revised, so that with each cell division and after marrow injury, the daughter stem cell is not identical to the parent stem cell in terms of replicative capacity. This concept is important when attempting to define the nature of the hemopoietic defect in AA where there is failure of normal hemopoiesis. Both a deficiency and a dysfunction of hemopoietic progenitor (CD34+) cells occurs, and, with hematological recovery, numbers of mature progenitor cells can return to normal but a deficiency and a dysfunction remains at the level of the primitive progenitor cells (LTC-IC). AA patients also show an exaggeration of the normal pattern of telomere shortening, which may contribute to the markedly reduced replicative capacity of the stem cells. This may occur because of an increased loss of stem cells and committed progenitor cells by apoptosis, or by direct immune destruction. The pattern of recovery of hemopoietic progenitor cell numbers in patients with idiosyncratic AA is similar to that seen following injury to normal bone marrow after chemotherapy, but the exact mechanism behind the injury to the stem cells is poorly understood and likely to be very different.

References

Abkowitz, J. L., Persik, M. T., Shelton, G. H. et al. (1995) Behaviour of haematopoietic stem cells in a large animal. *Proceedings of the National Academy of Sciences of the USA*, **92**, 2031–5.

Albella, B., Segovia, J. C. and Bueren, J. A. (1997) Does the granulocyte-macrophage colony forming unit content in ex-vivo expanded grafts predict the recovery of the recipient leucocytes? *Blood*, **90**, 464–70.

Amenomori, T., Honda, T., Otaka, M. et al. (1988) Growth and differentiation of circulating haemopoietic stem cells with atomic bomb irradiation-induced chromosome abnormalities. *Experimental Hematology*, **16**, 849–53.

Andrews, R. G., Singer, J. W. and Bernstein, I. D. (1989) Precursors of colony forming cells in humans can be distinguished from colony forming cells by expression of the CD33 and CD34 antigens and light scatter properties. *Journal of Experimental Medicine*, **169**, 1721–31.

Bacigalupo, A., Figari, O., Tong, J. et al. (1992) Long term marrow cultures in patients with aplastic anaemia compared with marrow transplant recipients and normal controls. *Experimental Haematology*, **20**, 425–30.

Bacigalupo, A., Piaggio, G., Podesta, M. et al. (1993) Collection of peripheral blood haemopoietic progenitors (PBHP) from patients with severe aplastic anaemia (SAA) after prolonged administration of granulocyte colony stimulating factor. *Blood*, **82**, 1410–14.

Ball, S. E., Gibson, F. M., Rizzo, S. et al. (1998) Progressive telomere shortening in aplastic anemia. *Blood*, **91**, 3582–92.

Barrett, A. J., Faille, A., Balitrand, N. et al. (1979) Bone marrow culture in aplastic anaemia. *Journal of Clinical Pathology*, **32**, 660–5.

Capel, B., Hawley, R., Covarrubias, L. et al. (1988) Clonal contributions of small numbers of retrovirally marked haematopoietic stem cells engrafted in unirradiated neonatal W/W^{v} mice. *Proceedings of the National Academy of Sciences of the USA*, **86**, 4564–8.

Champion, K. M., Gilbert, J. G. R., Asimakopolos, F. O. et al. (1997) Clonal haemopoiesis in normal elderly women; implications for the myeloproliferative disorders and myelodysplastic syndromes. *British Journal of Haematology*, **97**, 920–6.

de Wynter, E. A., Coutinho, L. H., Pei, X. et al. (1995) Comparison of purity and enrichment of CD34+ cells from bone marrow, umbilical cord and peripheral blood (primed for apheresis) using five different separation systems. *Stem Cells*, **13**, 524–32.

de Wynter, E. A., Nadali, G., Coutinho, L. H. and Testa, N. G. (1996) Extensive amplification of single cells from CD34+ subpopulations in umbilical cord blood and identification of long-term culture initiating cells present in two subsets. *Stem Cells*, **14**, 566–76.

Fairbairn, L. J., Cowling, G. J., Reipert, B. M. and Dexter, T. M. (1993) Suppression of apoptosis allows differentiation and development of a multipotent haemopoietic cell line in the absence of added growth factors. *Cell*, **74**, 823.

Gale, R. E., Fielding, A. K., Harrison, C. N. and Linch, D. C. (1997) Acquired skewing of x-chromosome inactivation patterns in myeloid cells of the elderly suggests stochastic clonal loss with age. *British Journal of Haematology*, **98**, 512–19.

Gibson, F. and Gordon–Smith, E. C. (1990) Long term culture of aplastic anaemia bone marrow. *British Journal of Haematology*, **75**, 421–7.

Gibson, F. M., Scopes, J. and Gordon–Smith, E. C. (1996) Regeneration of aplastic anaemia progenitor cells from 5-fluorouracil treated bone marrow in long term culture. *Experimental Haematology*, **24** [Suppl. 1], 209a.

Hara, H., Kai, S., Fushimi, M. et al. (1980) Pluripotent haemopoietic precursors in vitro (CFU-Mix) in aplastic anaemia. *Experimental Haematology*, **8**, 1165–71.

Holmberg, L. A., Seidel, K., Leisenring, W. et al. (1994) Aplastic anaemia: analysis of stromal cell function in long term marrow cultures. *Blood*, **84**, 3685–90.

Hotta, T., Kato, T., Maeda, H. et al. (1985) Functional changes in marrow stromal cells in aplastic anaemia. *Acta Haematologica*, **74**, 65–9.

Jimenez, G., Griffiths, S. D., Ford, A. M. et al. (1992) Activation of the beta-globulin focus control region precedes commitment to the erythroid lineage. *Proceedings of the National Academy of Sciences of the USA*, **89**, 10618.

Keller, G. and Snodgrass, R. (1990) Life span of multipotential haematopoietic stem cells in vivo. *Journal of Experimental Medicine*, **171**, 1407–18.

Kern, P., Heimpel, H., Heit, W. et al. (1977) Granulocytic progenitor cells in aplastic anaemia. *British Journal of Haematology*, **35**, 613–23.

Kusunoki, Y., Kodama, Y., Hirai, Y. et al. (1995) Cytogenetic and immunologic identification of clonal expansion of stem cells into T and B lymphocytes in one atomic-bomb survivor. *Blood*, **86**, 2106–12.

Lajtha, L. G. (1983) In *Stem cell concepts in stem cells: their identification and characterisation*, ed. C. S. Potten, pp. 1–11. London: Churchill Livingstone.

Larochell, A., Vormoor, J., Hanenberg, M. et al. (1996) Identification of primitive human haematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nature Medicine*, **2**, 1329–37.

Lord, B. I., Heyworth, C. M. and Testa, N. G. (1997) An introduction to primitive haematopoietic cells. In: *Haematopoietic lineages in health and disease*, ed. N. G. Testa, B. I. Lord and T. M. Dexter, pp. 1–27. New York: Marcel Dekker.

Maciejewski, J. P., Anderson, S., Katevas, P. et al. (1994) Phenotypic and functional analysis of bone marrow progenitor cell compartment in bone marrow failure. *British Journal of Haematology*, **87**, 227–34.

Maciejewski, J. P., Selleri, C., Sato, T. et al. (1995*a*) Increased expression of Fas antigen on bone marrow CD34+ cells of patients with aplastic anaemia. *British Journal of Haematology*, **1**, 245–52.

Maciejewski, J., Selleri, C., Anderson, S. et al. (1995*b*) Fas antigen expression on CD34+ human marrow cells is induced by interferon-γ and tumour necrosis factor-α and potentiates cytokine mediated haemopoietic suppression in vitro. *Blood*, **85**, 3183–90.

Maciejewski, J. P., Selleri, C., Sato, T. et al. (1996) A severe and consistent defect in marrow and circulating primitive haemopoietic cells (long term culture initiating cells) in acquired aplastic anaemia. *Blood*, **88**, 1983–91.

Marsh, J. C. W. (1996) Long-term marrow cultures in aplastic anemia. *European Journal of Haematology*, **57** [Suppl. 60], 75–9.

Marsh, J. C. W., Chang, J., Testa, N. G. et al. (1990) The haemopoietic defect in aplastic anaemia assessed by long term marrow culture. *Blood*, **76**, 1748–57.

Marsh, J. C. W., Chang, J., Testa, N. G. et al. (1991) In vitro assessment of marrow 'stem cell' and stromal cell function in aplastic anaemia. *British Journal of Haematology*, **78**, 258–67.

Marsh, J. C. W. (1996) Long-term bone marrow cultures in aplastic anaemia. *European Journal of Haematology*, **57** [Suppl. 60], 75–9.

McArthur, G. A., Rohrschneider, L. R. and Johnson, G. R. (1994) Induced expression of c-fms in normal haematopoietic cells shows evidence for both conservation and lineage restriction of signal transduction in response to macrophage colony-stimulating factor. *Blood*, **83**, 972.

McArthur, G. A., Longmore, G. L., Klingler, K. and Johnson, G. R. (1995) Lineage-restricted recruitment of immature haematopoietic cells in response to erythropoietin after normal haematopoietic cell transfection with erythropoietin receptor. *Experimental Haematology*, **23**, 645.

Metcalf, D. (1988) In *The molecular control of blood cells*. London: Harvard University Press.

Nissen, C., Wodmar-Filipowicz, A., Slanicka-Krieger, M. et al. (1995) Persistent growth impairment of bone marrow stroma after antilymphocyte globulin treatment for severe aplastic anaemia and its association with relapse. *European Journal of Haematology*, **5**, 255–61.

Novotski, N. and Jacobs, P. (1995) Immunosuppressive therapy in bone marrow aplasia: the stroma functions normally to support haemopoiesis. *Experimental Haematology*, **23**, 1472–7.

Ogawa, M. (1993) Differentiation and proliferation in haematopoietic stem cells. *Blood*, **81**, 2844.

Osawa, M., Hanada, K., Hamada, H. and Nakauchi, H. (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoeitic stem cell. *Science*, **273**, 242–5.

Pettengel, R., Luft, T., Henschler, R. and Testa, N. G. (1994) Direct comparison by limiting dilution analysis of long-term culture initiating cells in human bone marrow, umbilical cord blood and blood stem cells. *Blood*, **84**, 3653–9.

Pflumio, F., Izac, B., Kats, A. et al. (1996) Phenotype and function of human haematopoietic cells engrafting immune-deficient CD17-severe combined immunodeficiency mice and non-obese-diabetic-severe combined immunodeficiency mice after transplant of human cord blood mononuclear cells. *Blood*, **88**, 3731–40.

Philpott, N. J., Scopes, J., Marsh, J. C. W. et al. (1995) Increased apoptosis in aplastic anaemia bone marrow progenitor cells: possible pathophysiological significance. *Experimental Haematology*, **23**, 1642–8.

Ploemacher, R. (1994) Cobblestone area forming cell (CAFC) assay. In *Culture of haematopoietic cells*, ed. R. I. Freshney, I. B. Pragnell and M. G. Freshney, pp. 1–21. New York: Wiley–Liss.

Podesta, M., Piaggio, G., Frassoni, F. et al. (1998) The assessment of the haemopoietic reservoir after immunosuppressive therapy or bone marrow transplantation in severe aplastic anaemia. *Blood*, **91**, 1959–65.

Ratajczak, M. Z. and Gewirtz, A. M. (1995) The biology of haematopoietic stem cells. *Seminars in Oncology*, **22**, 210–17.

Schrezenmeier, H., Jenal, M., Herrmann, F. et al. (1996) Quantitative analysis of cobblestone area forming cells in bone marrow of patients with aplastic anaemia by limiting dilution assay. *Blood*, **88**, 4474–80.

Scopes, J., Bagnara, M., Gordon–Smith, E. C. et al. (1994) Haemopoietic progenitor cells are reduced in aplastic anaemia. *British Journal of Haematology*, **86**, 427–30.

Scopes, J., Daly, S., Atkinson, R. et al. (1996) Aplastic anaemia: evidence for dysfunctional bone marrow progenitor cells and the corrective effect of granulocyte colony stimulating factor. *Blood*, **87**, 3179–85.

Spangrude, G. J. (1994) Biological and clinical aspects of haematopoietic stem cells. *Annual Review of Medicine*, **45**, 93–104.

Testa, N. G., Lord, B. I. and Shore, N. A. (1972) The in vivo seeding of haemopoietic colony forming cells in irradiated mice. *Blood*, **40**, 654–61.

Testa, N. G., de Wynter, E. A. and Weaver, A. (1996) The study of haemopoietic stem cells in patients: concepts, approaches and cautionary tales. *Annals of Oncology*, **7** [Suppl. 2], 5–8.

Testa, N. G., de Wynter, E. and Hows, J. (1999) Haemopoietic stem cells as targets for genetic manipulation: concepts and practical approaches. In: *Haematopoiesis and gene therapy*, ed. L. Fairbairn and N. G. Testa, pp.1–12. London: Plenum Press.

To, L. B., Haylock, D. N., Simmons, P. J. and Juttner, C. A. (1997) The biology and clinical uses of blood stem cells. *Blood*, **89**, 2233–58.

Turhan, A. G., Humphries, R. K., Phillips, G. L., Eaves, A. C. and Eaves, C. J. (1989) Clonal hematopoiesis demonstrated by X-linked DNA polymorphisms after allogeneic bone marrow transplantation. *New England Journal of Medicine*, **320**, 1655–61.

Turner, C. W., Yeager, A. M., Waller, E. K., Wingard, J. R. and Fleming, W. H. (1996) Engraftment potential of different sources of human haematopoietic progenitor cells in BNM mice. *Blood*, **87**, 3237–44.

Vaziri, H., Dragowska, W., Allsopp, et al. (1994) Evidence for a mitotic clock in human haematopoietic stem cells; loss of telomeric DNA with age. *Proceedings of the National Academy of Sciences of the USA*, **91**, 9857–60.

Vormoor, J., Lapidot, T., Pflumio, F. et al. (1994) Immature human cord blood progenitors engraft and proliferate to high levels in severe combined immunodeficiency. *Blood*, **83**, 2489–97.

Weaver, A., Ryder, W. D. J. and Testa, N. G. (1997) Measurement of long term culture initiating cells (LTC-ICs) using limiting dilution: comparison of endpoints and stromal support. *Experimental Haematology*, **25**, 1333–8.

Wynn, R. F., Cross, M. A., Hatton, C. et al. (1998) Accelerated telomere shortening in young recipients of allogeneic bone-marrow transplants. *Lancet*, **351**, 178–81.

2

Cytokine abnormalities in aplastic anemia

Seiji Kojima

Nagoya University School of Medicine, Japan

Introduction

The term aplastic anemia (AA) encompasses a group of stem-cell disorders characterized by peripheral-blood pancytopenia and hypocellular bone marrow. Although the exact mechanisms responsible for its pathogenesis are unknown, possible causes include a primary stem-cell defect, immune-mediated inhibition of hemopoiesis, and an abnormal bone marrow microenvironment (Camitta et al; 1982; Young and Maciejewski, 1997). Normal hemopoiesis is sustained by interactions between hemopoietic stem cells, cells of the bone marrow microenvironment, and cytokines produced by these cells. These cytokines are essential for the viability, proliferation, and differentiation of hemopoietic stem cells. In vitro evidence for the existence of a supporting microenvironment in hemopoiesis comes from the development of a long-term bone marrow culture (LTBMC) system (Dexter et al., 1977; Gartner and Kaplan, 1980). LTBMC is composed of confluent layers of marrow-adherent cells including fibroblasts, endothelial cells, adipocytes, and macrophages. LTBMC forms an in vitro model of the bone marrow microenvironment. Marrow stromal cells are thought to exert their regulatory role in hemopoiesis, at least in part, by the production of certain cytokines. Monolayer cultures of marrow-adherent cells have been shown to produce a variety of cytokines including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), and stem cell factor (SCF), either constitutively or after stimulation by interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) (Kaushansky et al., 1988; Linenberger et al., 1995; Schadduk et al., 1983).

Several earlier studies showed elevated circulating levels of colony-stimulating activity in patients with AA (Nissen et al., 1985; Yen et al., 1985). These studies employed bioassays to measure colony-stimulating activity and therefore detected the combined stimulatory effects of various cytokines. The recent

development of sensitive radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) has made it possible to measure specific cytokines even at low levels. To clarify the role of cytokines in the pathophysiology of AA, I will summarize here the circulating levels and production of various cytokines by peripheral mononuclear cells and marrow stromal cells in patients with AA.

Erythropoietin (EPO)

EPO is a glycoprotein produced by cells adjacent to the proximal renal tubules (Erslev, 1991; Kranz, 1991). In the bone marrow, EPO binds to and activates specific receptors on erythroid progenitor cells to regulate the production of red blood cells (RBCs). Under normal steady-state conditions, 10 to 20 mIU/ml of plasma EPO induces the production of enough RBCs to replace senescent cells in the RBC mass, and to maintain a flow of oxygen to the renal oxygen sensor which is sufficient to ensure baseline levels of EPO synthesis. Reduced flow of oxygen to the kidneys increases the rate of EPO synthesis exponentially, such that at a packed cell volume of less than 0.20, the plasma levels of EPO increase 100-fold or more (Erslev et al., 1980).

Several studies have shown highly elevated levels of EPO in patients with AA (Gaines Das et al., 1992; Schrezenmeier et al., 1994; Urabe et al., 1992). We have also measured plasma EPO levels in 75 patients with acquired AA. (Figure 2.1, Kojima et al., 1995). The median hemoglobin concentration was 7.2g/dl with a range of 3.8 to 14.2g/dl, and the median plasma EPO level was 2720 mIU/ml with a range of 103 to 19,800 mIU/ml. The normal range for plasma EPO was 18.3 ± 3.5 mIU/ml. Overall, there was an inverse relationship between the level of plasma EPO and the hemoglobin concentration. Plasma EPO levels varied tremendously among patients with the same degree of anemia. Two factors relevant to this variation were the etiology of AA and the interval between diagnosis and blood sampling. EPO levels in patients with posthepatitis AA were significantly lower than those in patients with idiopathic AA. Higher EPO concentrations were found in patients with a longer duration of disease.

We studied serial changes in plasma EPO levels in patients who showed an erythropoietic response following immunosuppressive therapy or bone marrow transplantation (BMT). A decrease in plasma EPO levels was noted in all erythroid responders. For any given degree of anemia, the plasma EPO level was lower in patients of the BMT group than in patients receiving immunosuppressive therapy. Furthermore, plasma EPO levels were inappropriately decreased in more than half of the patients who received BMT compared with iron-deficient patients. Cyclosporin probably caused the decreased plasma EPO levels in BMT patients.

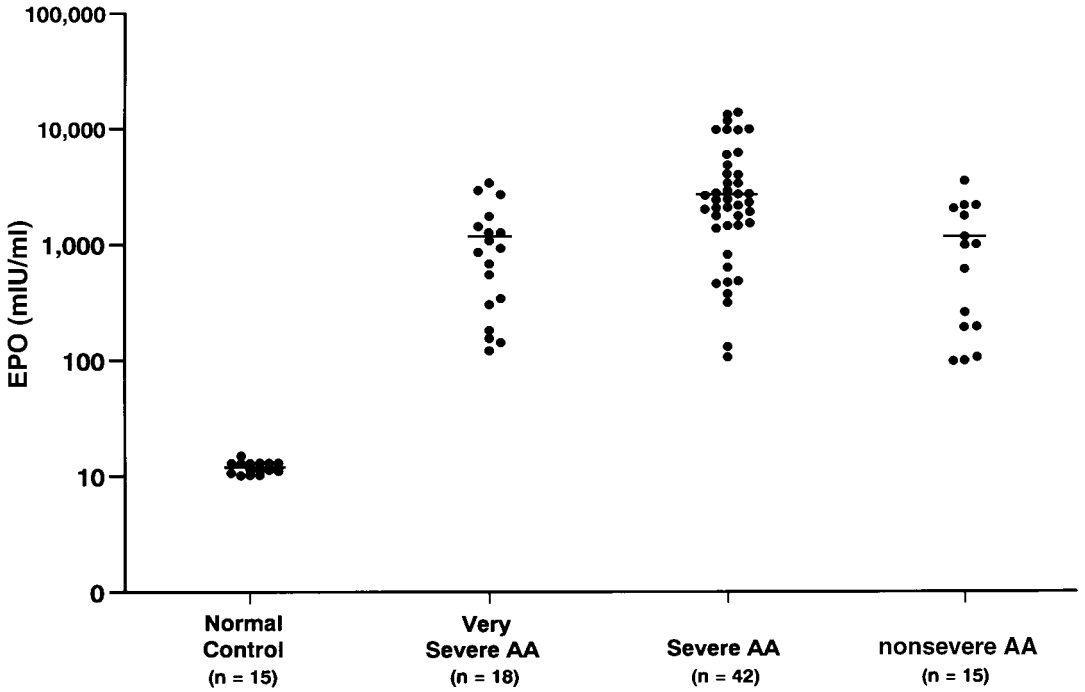

Figure 2.1. Plasma concentrations of erythropoietin (EPO) in 75 patients with aplastic anemia (AA) and 15 normal controls. Each horizontal bar indicates the mean value in the group.

Granulocyte colony-stimulating factor (G-CSF)

G-CSF is a glycoprotein that selectively and specifically stimulates the proliferation and differentiation of neutrophil precusors by binding to a specific cell surface receptor. G-CSF also activates several functions of mature neutrophils, including mobility, adherence, phagocytosis, bacterial killing, and superoxide release. Monocyte/macrophages and bone marrow stromal cells are known to produce G-CSF (Demetri and Griffin, 1991; Lieschke and Burgess, 1992). In recent clinical trials using recombinant human (rh) G-CSF to treat AA, a transient increase in neutrophil counts was induced in the majority of patients (Kojima and Matsuyama, 1994; Kojima et al., 1991).

Although previous studies have shown elevated levels of G-CSF in the sera of some AA patients, serum levels of G-CSF are below the lower limits of detection in a considerable percentage of AA patients or normal controls (Motojima

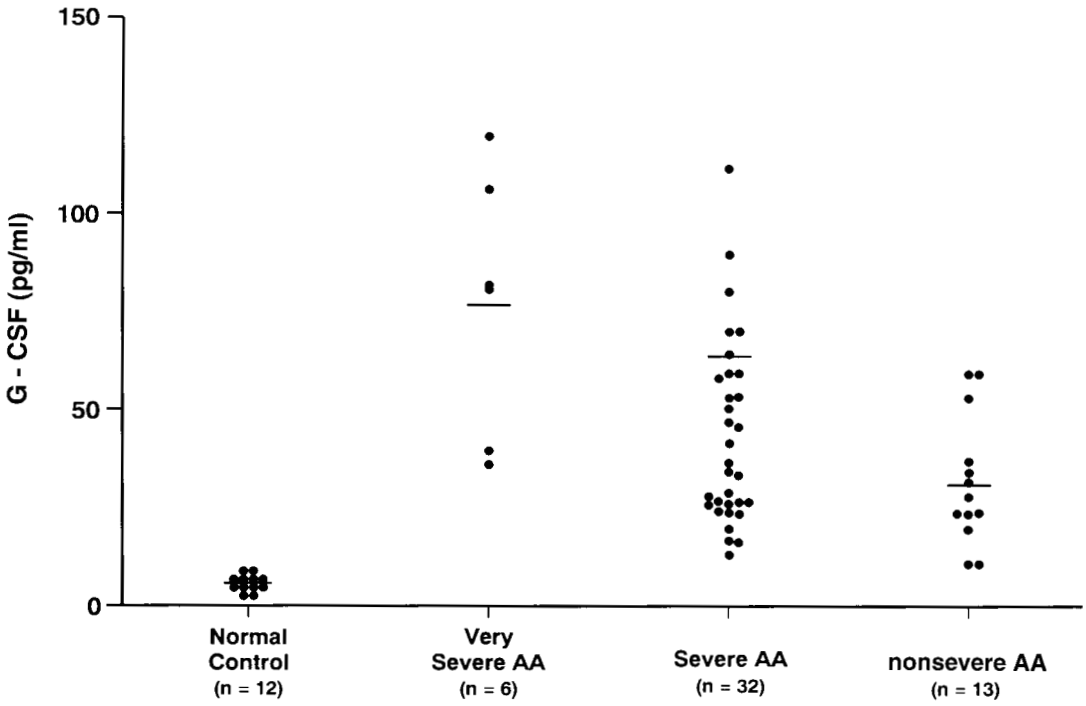

Figure 2.2. Plasma concentrations of granulocyte colony-stimulating factor (G-CSF) in 51 patients with aplastic anemia and no sign of infection and 12 normal controls. Each horizontal bar indicates the mean value in the group.

et al., 1989; Omori et al., 1992; Watari et al., 1989). We measured endogenous plasma G-CSF levels in 68 patients with acquired AA (51 of whom had no sign of infection) by a chemiluminescent immunoassay (Figure 2.2, Kiriyama et al., 1993; Kojima et al., 1996). The minimum detection limit of this assay (0.5 pg/ml) was sufficient to determine G-CSF levels in all AA patients and normal controls. In 51 AA patients who had no signs of infection, plasma G-CSF levels were significantly elevated compared with the normal control group (42.1 ± 23.6 pg/ml versus 6.8 ± 1.7 pg/ml, $P < 0.001$). Plasma G-CSF levels were also significantly higher in the 17 AA patients with signs of infection compared with the 51 AA patients without obvious infection (1211.8 ± 1850.4 pg/ml versus 42.1 ± 23.6 pg/ml, respectively, $P < 0.01$). There was a significant negative correlation between plasma G-CSF levels and absolute neutrophil counts (ANC) in AA patients with no signs of infection. The 51 patients were grouped on the basis of sex, age, interval between diagnosis and time of study entry, and type of therapy at the time of study entry. None of these variables was significantly associated with differences in mean plasma G-CSF levels between subgroups.

Plasma G-CSF levels were determined before and 2–3 months after immunosuppressive therapy or BMT. In the 13 patients undergoing BMT, the mean ANC increased from $(0.77 \pm 0.48) \times 10^9$/l to $(1.98 \pm 1.04) \times 10^9$/l following BMT. The mean ANC also increased from $(0.50 \pm 0.23) \times 10^9$/l to $(1.64 \pm 1.20) \times 10^9$/l in ten responders to immunosuppressive therapy. Although a decrease in the plasma G-CSF concentration was observed in all patients who achieved self-sustaining hemopoiesis following BMT or immunosuppressive therapy, it was lower in patients undergoing BMT as compared with those receiving immunosuppressive therapy for any given degree of neutropenia.

Thrombopoietin (TPO)

TPO is a cytokine that supports megakaryocyte colony formation, increases megakaryocyte size and ploidy, and is the most important regulator of platelet production (Kaushansky, 1995). TPO has been identified as a ligand for c-Mpl, and full-length complementary deoxyribonucleic acid sequences (cDNAs) encoding human TPO have been cloned and sequenced (Bartley et al., 1994; Lok et al., 1994; de Sauvage et al., 1994; Sohma et al., 1994; Wendling et al., 1994). The predominant sites of TPO production are the liver and, to a lesser degree, the kidney, although the specific cells responsible for production are not known (Kaushansky, 1995). As TPO is the physiological regulator of platelet production, its circulating levels vary with blood platelet counts.

There are only a few studies that have examined endogenous levels of TPO in patients with AA (Emmons et al., 1996; Marsh et al., 1996). We measured endogenous plasma levels of TPO in 76 patients with acquired AA using ELISA (Figure 2.3; Kojima et al., 1997*b*; Tahara et al., 1996). In patients with AA, the median platelet count was 18×10^9/l with a range of 1×10^9/l to 110×10^9/l. The mean plasma TPO level was 29.7 ± 12.9, 21.3 ± 10.6, 16.6 ± 7.4, and 1.3 ± 0.3 fmol/ml in patients with very severe AA, severe AA, nonsevere AA, and normal controls, respectively. The mean plasma TPO level was significantly higher in patients with AA than in normal controls ($P < 0.001$), and there was a significant negative correlation between plasma TPO levels and platelet counts in 54 AA patients who had not received any platelet transfusions prior to sampling. We did not find any factors responsible for the variation in plasma TPO levels.

We studied serial changes in plasma TPO levels in patients who showed an increase in their platelet counts following immunosuppressive therapy or BMT. In 14 patients undergoing BMT, the mean platelet count increased from $(13 \pm 5) \times 10^9$/l to $(169 \pm 26) \times 10^9$/l following BMT. A decrease in TPO level was observed in all patients. However, although the mean platelet count increased from $(13 \pm 6) \times 10^9$/l to $(55 \pm 35) \times 10^9$/l in ten responders to immunosuppressive

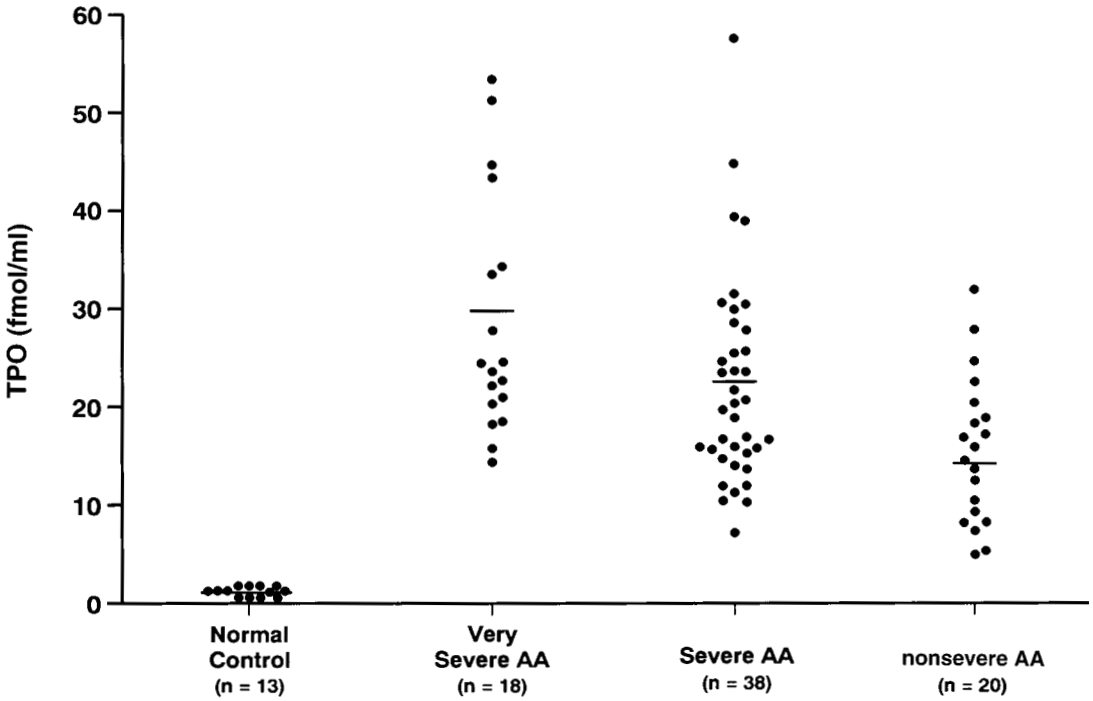

Figure 2.3. Plasma concentrations of thrombopoietin (TPO) in 76 patients with aplastic anemia and 13 normal controls. Each horizontal bar indicates the mean value in the group.

therapy, a decrease in TPO level was observed in only half of them. These findings differed from our previous studies in which decreased plasma EPO and G-CSF levels were observed in all AA patients who achieved self-sustaining hemopoiesis after treatment. Even in patients who respond to immunosuppressive therapy, mild thrombocytopenia and decreased megakaryocyte numbers in the bone marrow are present in the majority. These findings may explain why plasma TPO levels remain high in AA patients who achieved self-sustaining hemopoiesis and further support the model that plasma TPO levels are regulated not only by blood platelet count but also by the number of megakaryocytes or megakaryocyte progenitors in the bone marrow.

Stem cell factor (SCF)

SCF, the ligand for the c-Kit receptor, is produced mainly by marrow stromal cells and has been implicated in the maintenance of an optimal hemopoietic environment (Huang et al., 1990; Martin et al., 1991; Williams et al., 1990; Zsebo et al., 1990). It is biologically active in both a soluble and a membrane-bound

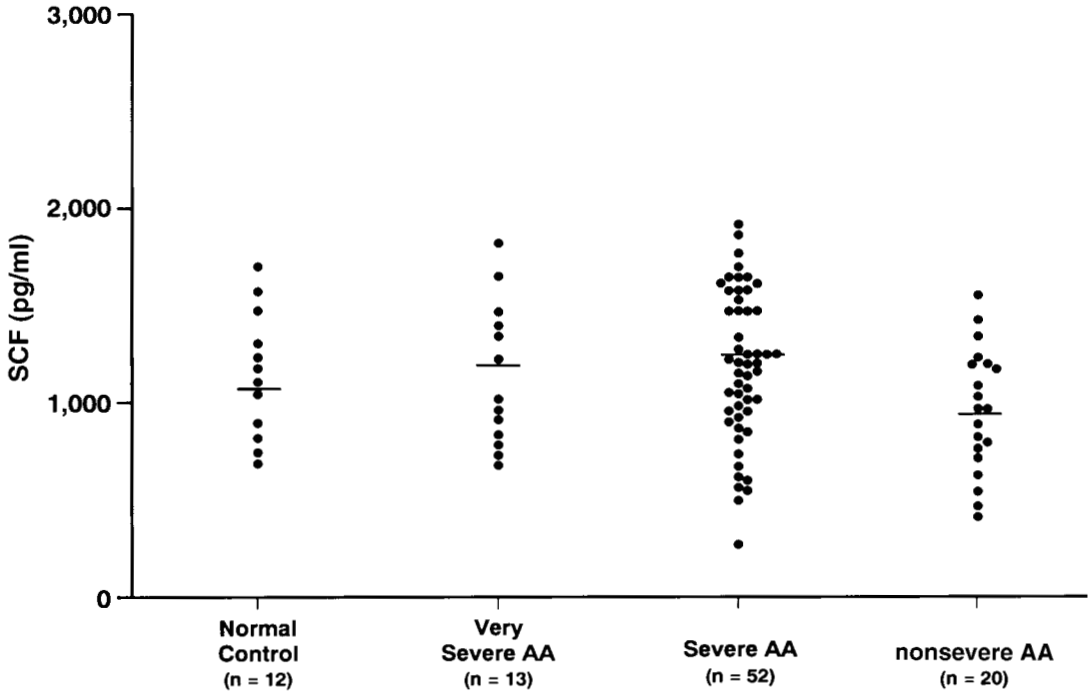

Figure 2.4. Plasma concentrations of soluble stem cell factor (SCF) in 85 patients with aplastic anemia and 12 normal controls. Each horizontal bar indicates the mean value in the group.

form (Anderson et al., 1990). In vitro, SCF alone fails to exhibit any hemopoietic colony-stimulating activity, but it acts synergistically with other more lineage-specific cytokines such as G-CSF, GM-CSF, IL-3, and EPO to augment colony formation by normal human bone marrow cells (McNiece et al., 1991; Tsuji et al., 1991). It can also partially reverse the reduced in vitro colony formation observed in patients with AA (Amano et al., 1993; Bagnara et al., 1992; Wodnar-Filipowicz et al., 1992).

To date, two studies have investigated levels of circulating SCF in patients with AA (Nimer et al., 1994; Wodnar-Filipowicz et al., 1993). Both studies demonstrated that the mean serum levels of SCF in AA patients are significantly lower than those in normal controls. Recently, we studied plasma levels of soluble SCF in 85 patients with AA (Figure 2.4, Kojima et al., 1997*a*). In contrast to previous studies, the mean SCF level in these 85 patients was 1098 ± 358 pg/ml, which was similar to that of normal controls (1160 ± 316 pg/ml); there was no significant correlation between peripheral blood counts and SCF levels. The mean SCF concentration in patients who received prednisolone with or without anabolic steroids at the time of sampling was significantly lower than in patients who did not receive these agents. We did not find any correlation between changes in SCF levels and the