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MICROENVIRONMENTAL INFLUENCES ON THE GROWTH OF NORMAL AND LEUKEMIC MYELOID CELLS IN THE RAT BONE MARROW

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The hemopoietic microenvironment of the bone marrow is an essential regulator of *in vivo* hemopoiesis. In addition to supporting the growth of normal blood cells, it also influences the growth of leukemia. This thesis describes the use of a rat model to examine three aspects of the function of the hemopoietic microenvironment. First, using a myeloid leukemia cell line (BNML), we showed that the pattern of growth of these cells differed in the bone marrow and spleen, that their presence was associated with a relocalization of normal hemopoietic stem cells from marrow to spleen, and that factors (yet to be defined) released from spleen cells altered the pattern, possibly to create a more permissive environment. Second, we showed that the "ST3" marker of marrow fibroblasts was associated with the Thy-1 molecule, and either directly or indirectly contributed to the *in vitro* adhesion reaction between marrow fibroblastoid cells and normal and leukemic myeloid precursors. Third, we showed that ectopic bony ossicles induced by subcutaneous implantation of recombinant human bone morphogenetic protein-2 contained marrow expressing the full range of hemopoiesis, including stem cells with a potential for long-term repopulation (demonstrated using a rat Y-chromosome specific DNA probe that we developed), and contained fibroblastoid cells differentiated to express the ST3 antigen in a manner similar to those from femoral bone marrow. These results provide further evidence for, although not final proof of, the hypothesis that the ST3 antigen participates in the function of the rat hemopoietic microenvironment, and points the way to future experiments on the interactions between stromal elements and normal and leukemic myeloid precursors.

RÉSUMÉ

Le microenvironnement hématopoïétique de la moëlle osseuse est un régulateur essentiel de l'hématopoïèse *in vivo*. En plus de soutenir la croisssance des cellules sanguines normales, il influence aussi celle de la leucémie. Cette thèse décrit l'utilisation d'un modèle de rat afin d'examiner trois aspects de la function du microenvironnement hématopoïétique. Premièrement, en utilisant une lignée de cellules leucémiques myéloïdes (BNML), nous avons demontré que le patron de croissance de ces cellules différait dans la moëlle osseuse et la rate, que leur présence état associée à une relocalisation des cellues hématopoïétique souches normales de la moelle osseuse à la rate et que des facteurs (qu'il reste a définir) libérés par des cellules spléniques altéraient ce patron, possiblement pour créer un environnement plus permissif. Deuxièmement, nous avons demontré que le marqueur de fibroblastes médullaires "ST3" était associé à la molécule Thy-1 et contributait, directement ou indirectement, à la réaction d'adhérence *in vitro* entre les cellules fibroblastoides de la moëlle osseuse et les précurseurs normaux et leucémiques. Troisièmement, nous avons demontré que des osselets ectopiques induits par l'implantation sous-cutanée de la protéine-2 morphogénétique recombinante de l'os humain contenaient de la moëlle exprimant toute l'étendue de l'hématopoïèse, incluant des cellules souches avec un potentiel de repopulation a long terme (demontré en utilisant un marqueur d'ADN spécifique au chromosome Y du rat que nous avons mis au point), et contenainet de cellules fibroblastoides différenciées pour exprimer l'antigène ST3 de façon similaire a celles de la moëlle osseuse fémorale. Ces résultats fournissent une manifestation supplémentaire, sans toutefois être la preuve finale, de l'hypothèse voulant que l'antigène ST3 participe à la function du microenvironnement hématopoïétique du rat et ils dirigent ves des expériences futures sur les interactions entre les éléments du stroma et les précurseurs myéloïdes normaux et leucémiques.

ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to my thesis supervisor, Dr. A.K. Sullivan for his guidance, patience, and encouragement during my graduate studies. I am also especially deeply grateful to Dr. N. Beauchemin for her expert advice on molecular biology, and for her support in providing me with an excellent environment in which to perform the cloning of the rat Y-chromosome probe.

I would like to express my gratitude to Dr. H.L. Goldsmith for accepting me into the Experimental Medicine program. Also, I am deeply grateful to Drs. G. Price, P. Laneuville, H. Bennett, E. Daniels, P. Brodt, and N. Dainiak for their criticism, helpful advice, and encouragement.

I am greatly indebted to Ms. K. Ceccarelli, Ms. H. Disco, Ms. C. Turbide, Mr. J. Albanese, Dr. Ming Ru, and Dr. P. Nedellec for their technical assistance and friendship. I am also greatly indebted to Ms. L. Welch for her secretarial help.

Special deep gratitude goes to my family, especially my mother and my husband, for their understanding and support.

PREFACE

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This thesis contains eight "Chapters". Following the English and French abstracts is a general introduction (Chapter 1); Chapters 2 through 5 contain the experimental results, presented as either published or submitted manuscripts, each with its own Abstract, Introduction, Material and Methods, Results, Discussion, and References, and connecting prefaces; then follows a general discussion and claims to originality (Chapters 6-7). The list of references (Chapter 8) cited in the introductory literature review appears last.

The published or submitted manuscripts are as follows:

Chapter 2. An J, Albanese J, Sullivan AK. The pattern of progression of myeloid leukemia (BNML) differs in rat bone marrow and spleen. Leukemia Research (Submitted for publication).

Chapter 3. An J, Disco H, Albanese J, and Sullivan AK. Expression of a Thy-1 like protein on rat marrow stromal cells and its possible role in adhesion of myeloid precursors. British Journal of Haematology (Submitted for publication).

Chapter 4. An J, Rosen V, Cox K, Beauchemin N, Sullivan AK. Recombinant human bone morphogenetic protein-2 induces a hemopoietic microenvironment in the rat that supports the growth of stem cells. Experimental Hematology 24:768-775, 1996

Chapter 5. An J, Beauchemin N, Albanese J, and Sullivan AK. Use of a DNA probe specific for the rat Y-chromosome to detect male-derived cells. Journal of Andrology (Submitted for publication).

In Chapter 2. the candidate was responsible for the all of the experiments; Mr. J. Albanese assisted with performing the flow cytometry. In Chapter 3, the candidate was responsible for the major part of the experiments. Mr. J. Albanese and Ms. H. Disco assisted with performing the western blot analyses. In Chapter 4, the bone morphogenetic protein was a generous donation from the laboratory of Dr. V. Rosen, Genetics Institute, Cambridge, Massachusetts, USA. In Chapter 5, the candidate was responsible for most of the experiments, performed in Dr. Beauchimin's laboratory under her advice; experiments establishing the efficiency and specificity of the rat Y-chromosome in tracking male-derived cells were performed by Mr. Albanese.

TABLE OF CONTENTS

ABBREVIATIONS

BDGF	Bone derived growth factor
BMP	Bone morphogenetic protein
BMT	Bone marrow transplantation
BNML	Brown Norway rat acute myelocytic leukemia
CAM	Cellular adhesion molecule
CDGF	Cartilage derived growth factor
CFU	Colony forming unit
CSF	Colony stimulating factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbant assay
FGF	Fibroblast growth factor
HM	Hemopoietic microenvironment
HST	Hemopoietic stem cells
IL	Interleukin
LTBMC	Long-term bone marrow cell culture
LTCIC	Long-term culture initiating cells
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
MPA	Mycophenolic acid
MW	Molecular weight
PDGF	Platelet derived growth factor
PKC	Protein kinase C
PTH	Parathyroid hormone
RGD	Arginine-Glycine-Aspartic acid
rhBMP	Recombinant human bone morphogenetic protein
S.C.	Subcutaneous
SCID	Severe combined immunodeficient
SF	Steel factor
SGF	Skeletal growth factor
TBI	Total body irradiation
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VGF	Vaccinia growth factor
AGM	Aorta gonad mesonephrons

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX

CHAPTER 5

LIST OF TABLES

CHAPTER 3

CHAPTER 4

CHAPTER 1

INTRODUCTION

The bone marrow stroma is a major component of the production of blood cells, and investigation into the mechanisms involved have led to the notion of a "hemopoietic microenvironment" (HM) in both the embryo and the adult (Wolf, 1968; Dexter, 1982; Tavassoli, 1991; Eaves, 1991; Moore, 1991; Greenberger, 1991; Zipori, 1992; Yoder, 1994, Verfaillie, 1994). Because the relationships among hemopoietic stem cells (HSCs), stromal cells, extracellular matrix (ECM), and humoral factors are complex, several investigators have sought to develop *in vitro* models, including the long-term bone marrow culture (LTBMC) system, in an attempt to dissect the essential elements. However, it is not well understood how all the constituent parts of the HM create a functional architecture or interact with developing hemopoietic cells. The work described herein aimed to use ***both in vivo and in vitro*** rat models to explore some of these relationships, using three experimental systems that could lead to further studies on mechanisms of hemopoietic regulation.

OBJECTIVES.

1. Assuming that the bone marrow, spleen, and thymus, have differentiated their stromal components to serve different tasks, test whether the pattern of *in vivo* growth of myeloid leukemia cells (BNML) might provide evidence for differential regulation.

2. Using both BNML cells and normal myeloid precursors, test whether an antigen marker for marrow fibroblasts ("ST3") contributes to their capacity to bind hemopoietic cells.

3. Use the ectopic marrow that forms after subcutaneous implantation of human recombinant bone morphogenetic protein-2 (rhBMP-2) as a model for *de novo* differentiation of marrow stroma in an adult mammal; test whether the stromal fibroblasts that develop express the ST3 antigen.

1. GENERAL REVIEW.

The bone marrow (BM), a mesodermal derivative, first forms during development as a HM which is then colonized by pluripotent stem cells; it is the only physiologic organ of hemopoiesis during the adult life of mammals (Werts, 1980; Tavassoli, 1983; Trentin, 1989; Fan, 1990). Evidence supporting the fact that the HM actually regulates the production of blood cells came from the following studies.

First, in mature hemopoietic tissues, the HM may differ in areas where one lineage or stage of maturation predominates, as suggested by the classic experiments in which intact bone marrow was implanted into the spleen (Wolf, 1968), and by kinetic gradient studies in which the distribution of CFU-S, CFU-C, and CFU-F was determined by using tritiated thymidine assays (Gong, 1978; Yang, 1984; Lord, 1990). Secondly, *in vitro*, stromal cells derived from the HM of bone marrow are required for the maintenance, proliferation, and differentiation of hemopoietic cells in the LTBMC (Dexter, 1982). Direct association between hemopoietic cells and stromal elements may mimic stroma-dependent hemopoiesis *in vivo* (Dexter, 1982; Greenberger, 1984). An increasing number of growth factors and adhesion molecules expressed by BM stromal cells are suggested to contribute to the regulation of hemopoiesis, some of which exhibit lineage and stage specific effects under defined *in vitro* conditions (Tsai, 1987; Cashman, 1990; Williams, 1991; Zipori, 1992; Teixido, 1992; Campbell, 1992). Thirdly, clinical studies support the notion that stromal cells of the HM play an important role in successful stem cell transplantation by creating the conditions necessary for stem cell seeding, stabilization, self-renewal and differentiation (Tavassoli, 1990; Moore, 1991).

Such experiments led to the "niche" hypothesis, which proposes that the spatial relationships of HSCs and progenitors to the stroma determine the response to growth and differentiation signals (Schofield, 1978; Lambertsen, 1984; Dorshkind, 1990; Lord, 1990). These signals further influence the expression of either nuclear proteins (GATA-2, GATA-1, PU.1, c-Myb, c-myc, p53, BCL-2, etc.) or cellular factors that determine and maintain the lineage program of hemopoietic cells (Schwartzman, 1993, Orkin, 1995). Many of the relationships among stromal cells, hemopoietic cells, ECM, and humoral

regulators are still undefined. An appropriate *in vivo* model would facilitate the study of these relationships at a multidimensional and functional level.

Several approaches have attempted to establish that the "HM" supports hemopoiesis, such as implantation of bone with or without marrow, marrow-derived stromal cells, or their ECM at ectopic sites (Friedenstein, 1974; Fan, 1990; Sadovnikova, 1991). However, these techniques are complex, the implants at the site are difficult to manipulate, and the marrow tissue can not be sustained for an extended period of time. The *de novo* ossicle induced by subcutaneous implantation of decalcified crude bone matrix powder or BMPs (Reddi, 1980; McCarthy, 1984; Wozney, 1988) provides a means by which the problems mentioned above can be overcome, and would allow study of the entire process of bone/bone marrow development in response to a single protein in an adult mammal.

BMPs are members of the TGF-β superfamily with a molecular weight of 26-31 kDa (Kingsley, 1994). The osteogenic activity of BMPs acts across species, as demonstrated by the fact that bovine, porcine, and human products are active in rats (Wozney, 1988). The events that follow subcutaneous implantation are reminiscent of embryonic long bone formation, which includes development of cartilage and subsequent calcification, vascular invasion, and finally remodelling of bone and growth of bone marrow. The hemopoiesis and the associated HM in this model is not well characterized. Such isolated "organoids" provide a new experimental model with which to study the developmental process of the regenerated *de novo* hemopoietic tissue in an adult, and to study how the marrow stroma forms and creates a functional microenvironment permissive to hemopoiesis.

2. HUMORAL REGULATION OF HEMOPOIESIS.

Four modalities of signalling have been proposed to play an important role in the regulation of hemopoiesis (Moore, 1991; Greenberger, 1991; Massagué, 1993). (1) Cell-cell contacts, which can be highly specific as in the case of recognition through the major

histocompatibility complex (MHC), or less specific when contacts are mediated by adhesion molecules; (2) short-range signalling by substances such as cytokines, lymphokines, and interleukins directed to specific receptors on cell membranes; (3) long-range signalling by hormones produced by endocrine glands which act at sites distant from the target organ(s); and (4) the ECM of the microenvironment constituted by stromal cells.

2.1. Growth Factors

The establishment of cell culture systems to measure the clonal growth of hemopoietic cells in liquid and semisolid medium led to the discovery of many potential regulatory molecules (Clark, 1987; Metcalf, 1990). Because they were identified initially in culture supernatants from various sources and stimulated the proliferation and differentiation of hemopoietic progenitors in short-term clonogenic assays, they were called "colony stimulating factors (CSFs)". Subsequently, other factors were noted to support the long-term survival of hemopoietic progenitors and stem cells in the LTBMC system (Eaves, 1991; Zipori, 1992; Du, 1994). Together, these proteins and peptides may be derived from different types of normal cells (e.g., lymphocytes, endothelial cells, fibroblasts, and monocytes) (Otsuka, 1988; Bagby, 1986; Kaushansky, 1988; Vellenga, 1988) or neoplastic cells (e.g., leukemic cells, etc.) (Young, 1987; Cozzolono, 1989; Wakamiya, 1989). At present, more than 20 humoral factors that influence *in vitro* hemopoiesis or hemopoietic cell function have been found, their genes cloned, and active recombinant molecules produced. Each of them is encoded by a single gene (Metcalf, 1990; Paul, 1990; Anderson, 1990; Kelleher, 1991; Hannum, 1994). Table 1 shows the major growth factors in the regulation of hemopoietic cell development *in vitro*. Most of them exert their effects on a wide range of target cells, and show overlapping and synergistic activities in different assays (Williams, 1992; Hiryama, 1994; Jacobsen, 1994). In spite of the overlapping action of these growth factors, the polypeptide chains of these proteins do not share significant amino acid homology, and also differ in secondary structure. The glycosylation of growth factors is not always necessary for their biological activity (Nicola, 1989). Of note is the clustering of genes for several growth

factors or their receptors. For example, the genes for IL-3, 4, 5, 9, GM-CSF, M-CSF, ECGF, c-fms, β-2 adrenergic receptor and CD14 are located on the same human chromosome 5q.

Table 1. Known hemopoietic growth factors

Factor	Target cell	Cellular source	MW (KD)	Gene location
GM-CSF	M,G,Eo, Meg, Multi	Mo,M,Fib,T	35	5q
G-CSF	G	Mo,M,Endo,Fib	20	17q
M-CSF	M	Mo,M,Fib,Epi,T	70	5q
EPO	E	Kidney peritubular cells	34	7q
IL-1	Multi	Mo,Mϕ,B,Fib, Dentric, Astro	17	2q
IL-2	T,B	Activated T	19	4q
IL-3	G,M,Eo,Meg,Mast, multi	Mo,T	28	5q
IL-4	B,T,E,Mast	Activated T	15	5q
IL-5	B,Eo	T	20	5q
IL-6	B,T,G,Multi	Mo,Mϕ,B,Epi, Dentric,Fib,Kerat	26	7p
IL-7	B,T	BM stroma	17.4	8q
IL-9	T,E	Leukocyte	40	5q
IL-11	Meg,B,Mast	PU-34 cell line	22	19q
KL	B,T,Mo,Mast, Multi	Stroma	18.5	NK
FLK-2	Multi	Thymic stroma	65	NK

Mϕ, macrophage; Mo, monocyte; G, granulocyte; B & T, lymphocyte; Eo, eosinophil; Meg, megakaryocyte; Multi, multipotential cell; E, erythrocyte; Endo, endothelial cell; Epi, epithelial cell; Fib, fibroblast cell; Kerat, keratocyte KL, *kit* ligand; FLK, fetal liver kinase; NK, not known.

2.2. *Growth inhibitors*

By analogy to hormone regulated systems, mature hemopoietic cells might be expected to produce molecules that feed back to the stem cell compartment to control the further production of mature cells. It is clear that certain blood cells, particularly macrophages and T cells, can produce cytokines that are potent growth inhibitors that can act on a wide range of targets (Axelrad, 1990; Moore, 1991; Pranell, 1995). In addition, these and possibly other inhibitors can be produced by mesenchymal cells of the marrow and other tissues (Moore, 1991; Wang, 1992). The inhibitory effects are observed at

almost all levels of hemopoietic progenitor development (Olofsson, 1987; Yu, 1987; Roberts, 1988; Del Rizzo, 1988; Keller, 1994) (Table 2).

Table 2. Properties of selected negative hemopoietic regulators

Name	Source	Hemopoietic target cells	Native MW (KD)
TGF-β	Platelets, Placenta, Kidney, Mϕ	CFU-GEMm/GM/M, BFU-E	25
MIP-1α	T, Mϕ, monocytes	HSCs, CFU-S	50-100
LIA[1]	Conditioned medium of normal BMCs, AML, CML, HL line	CFU-GM	500-600
HP & HP5b[2]	rat marrow, human granulocytes	CFU-S	590
NRP[3]	C57BL/6 (B6), mouse marrow, B6 pan marrow cell line	BFU-E	79
Inhibin[4]	Sertoli cells, granulosa cells	BFU-E CFU-E	32
SDKP[5]	Marrow, fetal liver, serum, placenta	CFU-S	487

1. Lund negative regulators; 2. Vienna-Bergen hemoregulatory peptide; 3. Toronto regulatory protein; 4. La Jolla negative regulator; 5. Ville Juif negative regulator.

For the most part, their mechanisms of action remain undefined (Pragnell, 1995). In steady state hemopoiesis, most stem cells are not proliferating. This may be viewed as either a passive process permitted by the absence of positive signals, or an active process induced by suppressive negative regulators. These factors could function by blocking, down-modulating, or otherwise reducing the function of receptors for positive regulators on hemopoietic cells. They might also interfere with signal transduction pathways, transcriptional factors, mRNA production/stability, or genes activated by positive regulators. The general view is that *in vivo* hemopoiesis is the sum of the balance between stimulation and inhibition (Moore, 1991; Zipori, 1992).

2.3. Receptors

Each of these regulatory factors are targeted to different cell surface or soluble receptors (Krystal, 1991; Heaney, 1996), which play an essential role in the transduction or modification of their unique constellation of signals. Recently, receptors for IL-2α, IL-2β, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, EPO, G-CSF) and GM-CSF have been isolated and their genes cloned (Hatakeyama, 1989; Itoh, 1987; Idzerda, 1990; Takaki, 1990; Yamasaki, 1988; Goodwin, 1990; Chang, 1994; Winkleman, 1990; Gearing, 1989; Dipersio, 1988). It is now apparent that several of them have structural homology with the growth hormone and prolactin receptor family of the larger cytokine receptor superfamily. Identification of these sequence homologies indicates a related 257-865 amino acid transmembrane domain, and an α-helical extracellular ligand-binding domain containing four conserved cysteine residues and a Trp-Ser-X-Trp-Ser motif. There is no sequence homology of the intracytoplasmic domains, although several are rich in proline and serine residues, which may be important to direct the specificity of signal transduction (Krystal, 1991). The end result of the receptor mediated signal transduction is the activation or inhibition of key enzymes (Table 3), alteration of cytoskeletal proteins, and expression of transcription factors and other DNA-binding proteins (Ihle, 1995).

Table 3. Key enzymes involved in receptor mediated signal transduction

Growth factors	Activation enzymes
M-CSF	Tyrosine Kinase
PDGF	Tyrosine Kinase
EGF	Tyrosine Kinase
Insulin	Tyrosine Kinase
MGF	Tyrosine Kinase (c-kit)
G-CSF	Serine-Threonine
GM-CSF	Adenylate cyclase (macrophages) Guanylate cyclase (neutrophils)
IL-2	Protein Kinase C
IL-3	Phosphorylase

Most hemopoietic cells carry only a few hundred cytokine receptors on their surface (Larsen, 1990). Under physiologic conditions, cytokines can stimulate maximal biological responses when 10% of receptors are occupied (Nicola, 1989). The function of CSF receptors can be trans-down modulated by each other, e.g., the binding of one CSF to its own receptor can result in a loss of available receptors for another CSF (Krystal, 1991). The distribution of growth factor receptors on different types of cells might explain the pleiotropic effects of cytokines and much of their apparent overlap in biologic function. In addition, soluble receptors, which can modify the concentration of cytokines, function as "hormones" in the regulation of hemopoiesis (Heaney, 1996).

3. STROMAL REGULATION OF HEMOPOIESIS.

The direct contact between hemopoietic cells and their associated stromal elements has been demonstrated *in situ* (Lichtman, 1981). The development of different hemopoietic lineages appears to be regionally localized within particular types of stromal cell niches of the bone marrow (Dorshkind, 1990). Optimal long-term proliferation of pluripotent progenitors occurs only when they are in direct contact with stromal cells, or are adhering to ECM proteins elaborated by stromal cells under defined *in vitro* conditions (Dexter, 1982). Intravenously infused hemopoietic progenitor cells in an irradiated animal, can home selectively to the appropriate niche within the bone marrow and begin to proliferate and differentiate (Tavassoli, 1990). Such stroma-dependent hemopoiesis differs from that characterized by short term clonogenic assays. The short-term clonogenic assays of sparse cells suspended in a liquid or semisolid culture system and stimulated by isolated cytokines may more closely represent events occurring in the circulation under emergency conditions, such as bacterial infections in peripheral organs and the blood stream. Conversely, steady state hemopoiesis occurring in blood-forming organs is regulated by stromal factors which include humoral regulators and cell-surface or ECM molecules that support stem cell renewal and direct cell positioning (Moore, 1991; Dainiak, 1991; Verfaillie, 1994; McGlave, 1994). However, it is not well understood how stromal elements of the HM interact with developing hemopoietic cells

in which to control or restrict them within specific microenvironments of the marrow.

3.1. Characterization of stroma dependent hemopoiesis

Most current understanding about stromal regulation of hemopoiesis has come from observations using the LTBMC system or established stromal cell lines that mimic certain aspects of the *in vivo* mechanisms (Dexter, 1982; Singer, 1987; Greenberger, 1991; Paul, 1991; Fraser, 1992). The major cellular components identified in the LTBMC include fibroblastoid cells, macrophages, endothelial cells, and adipocytes (Dexter, 1982). Stroma-dependent hemopoiesis is characterized by several features that include: (1) support of stem cell renewal; (2) reduction of mature cell output by restricting the activity of differentiation factors; (3) secretion of unique stimulators and inhibitors of hemopoiesis; and (4) expression of cell surface associated adhesion molecules (Moore, 1991; Greenberger, 1991; Zipori, 1992).

Stromal cells have been shown to express mRNA for M-CSF, GM-CSF, G-CSF, IL-1, IL-6, IL-7, IL-11, LIF, b-FGF, SCF, TNF, and TGF-β_2, but this expression does not correlate with the capacity to support myelopoiesis (Paul, 1990; Gabbianelli, 1990; Anderson, 1990; Caldwell, 1991; Wetzler, 1991). Some of these factors act directly on hemopoietic progenitors/ precursors, others act indirectly through accessary cells, and some exert both direct and indirect effects (Cashman, 1990). Positive or negative regulators are, in general, constitutively produced by stromal cells, as indicated by the observations that transcription and/or translation of related gene products are rapidly induced by cytokines such as IL-1 and TNF (Wetzler, 1991). A critical concentration of humoral hemopoietic regulators could accumulate at a local site by components of the ECM (Gordon, 1987; Roberts, 1988). Some of the ECM proteins as well as CAMs produced by marrow stromal cells, but not collagen type I, III, IV or laminin, can promote attachment of hemopoietic cells with lineage and stage specificity (Verfaillie, 1994).

3.2. Cellular components of the hemopoietic microenvironment

The bone marrow stroma includes several cellular subtypes, including endothelium, fibroblastoid/reticulum cells, adipocytes, macrophages (osteoclasts), and osteoblasts, each performing a special function.

3.2.1. Endothelial cells

Vascular endothelial (VE) cells are strategically located at the interface between the tissue and its circulation. They now are recognized to be diverse, performing distinct biological functions at different vascular sites and in different organs (Engelberg, 1989; Rafii, 1994). As part of the HM, VE cells play a role in regulating hemopoiesis, as well as controlling the selection of normal hemopoietic stem cells, leukemic cells, and metastatic cells from the circulation (Tavassoli, 1990; Glinsky, 1993). These processes in turn are modified by a variety of adhesion molecules, interleukins, chemotactic factors, inductive cytokines, and hemopoietic growth factors. Much evidence has confirmed that molecules such as endotoxin, bacterial proteins, and cytokines (e.g., IL-1β and TNF-α) can induce expression of genes for hemopoietic growth factors in VE cells. Factors released by activated endothelial cells include GM-CSF/G-CSF/M-CSF (Zsebo, 1988; Fibbe, 1989), IL-6 (May, 1989), PDGF (Hajjar, 1987), IL-1 (Warner, 1987), and the chemotactic factor IL-8 (Sica, 1990).

Anatomic consideration of bone marrow structure would indicate that the sinusoidal endothelium regulates the homing and release of hemopoietic cells to and from the extravascular hemopoietic space. Homing of progenitor cells to the marrow can be divided into two major steps (Aizawa, 1988). First, progenitor cells are recognized by and interact with the luminal surface of the endothelium. Second, they pass through the endothelial layer and enter the extravascular hemopoietic compartment. Recently, a homing protein expressed on murine hemopoietic progenitor cells (lectin, 110 KD membrane protein) has been identified (Tavassoli, 1990), but its ligand, which appears to be a glycan moiety of a membrane glycoprotein (37 KD) of the endothelium, has not been as well characterized (Shiota, 1993). Treatment of endothelial cells with

neuraminidase, galactosidase, and mannosidase can reduce or nearly abolish the homing of progenitors (Matsuoka, 1989).

3.2.2. Fibroblastoid cells (adventitial reticular cells)

Fibroblastoid cells of the marrow may function differently from those of other tissues in that they can induce bone formation in diffusion chambers (Friedenstein, 1970), maintain hemopoietic cells during *in vitro* culturing (Ottmann, 1991; Sitnicka, 1995), and support hemopoiesis when transplanted to extramedullary sites (e.g., beneath the renal capsule, Friedenstein, 1974). Ectopic transplants of red marrow reconstruct red marrow, whereas implants of yellow marrow reconstruct yellow marrow (Patt, 1982; Bainton, 1986). Thus, the weight of evidence supports the notion that stromal fibroblasts are capable of transferring the specific features of the microenvironment of the originating organ (Reddi, 1975; Tavassoli, 1983). The main source of fibroblastoid cells in the marrow are the adventitia of the sinusoid, arteries, nerve sheaths, or bone associated cells. Ultrastructural studies indicate that the sinus adventitial reticular cells may be the principal origin, although free fibroblastoid cells may appear in hemopoietic spaces (Weiss, 1976). The bone marrow includes a heterogenous population of fibroblastoid cells of which a subpopulation can be measured in clonogenic assays (CFU-F). They may also vary in their adhesive and osteogenic properties, as well as the ability to form a HM (Tavassoli, 1983). In LTBMC, fibroblast-appearing cells comprise most of the stromal elements (Tavassoli, 1982). Many humoral hemopoietic regulators or colony-stimulating activities are produced from established fibroblastoid cell lines (Kaushansky, 1988; Yang, 1988; Sullivan, 1989; Du, 1994; Linenberger, 1995). Fibroblastoid cells can be distinguished from the usual vascular endothelium and macrophage variants, by lacking von Willebrand factor (VIII-associated antigen), Fc, and complement receptors, although they may resemble them under the light microscope. The fibroblastoid cells produce and deposit the ECM, which forms a fine meshwork supporting hemopoietic cells within the extravascular space. In addition to collagen (type I, III, and IV) and other ECM, they express a variety of CAM's which have been linked to their physiologic functions (Verfaillie, 1994).

3.2.3. Macrophages

The marrow monocytes/macrophages are derived from hemopoietic precursors (Kurihara, 1990). In general, they are important mediators of host defense against invasion by foreign substances (Nathan, 1980; Sasada, 1980). They function to ingest and digest particles, and to secrete inflammatory cytokines that regulate other arms of the immune system. The osteoclast is a specialized macrophage derivative which plays an important role in bone remodelling in order to create a specialized marrow architecture (Rodan, 1990, Shevde, 1994). In a manner similar to endothelial and fibroblastoid cells, activated macrophages produce humoral factors that modify marrow activity, including G-CSF, GM-CSF, IL-1, IFN, and TNF (Weisbert, 1989). Unique cellular interactions include the erythroblastic island which consists of a central "nurse" macrophage surrounded by erythroid cells at various stages of maturation; such complexes may play a key role in the process of erythroid cell development, as well as digesting extruded nuclei.

3.2.4. Adipocytes

Marrow adipocytes differ from adipocytes elsewhere, and may be closely related to fibroblastoid cells. Although adipocytes are sparse in the femoral marrow of rat and mouse, they are more plentiful in larger mammals (Weiss, 1976; Brookoff, 1982; Tavassoli, 1983). Fibroblastoid cells derived from mouse and human marrow can undergo transformation to adipocytes *in vitro* (Sale, 1995). Conversely, adipocytes may also be able to pass through a lipolytic phase and transform into fibroblastoid cells under specialized conditions (Hoshino, 1995). Although they are present in both red and yellow marrow, they may differ both biochemically and functionally (Tavassoli, 1983). The differentiation of adipocytes is markedly influenced *in vitro* by exogenous growth factors such as TGF-β (an inhibitor of adipogenesis) and hydrocortisone (a stimulator of adipogenesis). Therefore, bone marrow stromal cells may represent a type of multipotent mesenchymal cell, capable of further differentiation into adipocytes. Moreover, this differentiation process, which is influenced by the age of the animal, can be reversed under certain conditions of hematologic stress.

3.2.5. Stromal stem cells

It has long been recognized that progenitor cells for cartilage, bone, fat, and fibrous tissues exist among the fibroblastic cells of the marrow stroma (Ashton, 1980; Banayahu, 1989; Mathieu, 1992), but their origin in the bone marrow is uncertain. In general, they are a heterogeneous population which have the common property of adherence to the substratum *in vitro* (Dexter, 1982; Tavassoli, 1983) and share a common embryogenic origin (Favaloro, 1990; Timens, 1990). Although there might be a common embryonic mesenchymal stem cell (Huang, 1992), definitive evidence is lacking as to whether a common stem cell exists in the adult mammal. The identity of these presumed mesenchymal stem cells, the cell stage-dependant changes that occur during their development, and the mechanisms of controlling their functions remain unknown.

3.3. Extracellular matrix (ECM) and cellular adhesion molecules

Normal and abnormal cell proliferation and differentiation often requires not only growth factors, but also certain adhesion proteins (e.g., ECM proteins, CAMs, and junctional proteins) (Ip 1990; Imhof, 1991; Glinsky, 1993; Birchmeier, 1994). Such cell-cell and cell-ECM interactions are involved in cell recognition, migration and localization during embryonic development, tissue organization, cell differentiation, inflammation, and metastasis (Glinsky, 1993; Tuckwell, 1993). A variety of outputs are possible from cell contact mediated signals, some of which are very rapid and do not require protein or DNA synthesis (e.g., changes in cell shape, cytoskeletal reorganization and motility). Others transduce long-term signals, which include phosphorylation of cytoskeletal components, translocation of signalling intermediates (e.g., proto-oncogenes and PKC), and induction of early-response genes (Damsky, 1991). Many ECM adhesion molecules are large, multichain glycoproteins, and contain the tripeptide arginine-glycine-aspartic acid (RGD) as their cell recognition site. Also, many of them contain domains with close or distant homology to EGF, TGF and VGF (Engel, 1991). Most of the CAMs are single chain glycoproteins expressed on the cell surface (Williams, 1988), and interact with

structurally-related molecules (integrins and others), cooperately with other growth factor-receptor systems, and mediate multidimensional adhesive interactions among multiple cells (Tuckwell, 1993).

3.3.1. Adhesion molecules mediate cell-cell and cell-ECM interactions.

Adhesion is of fundamental importance to a cell. It provides anchorage and signals for growth and differentiation. The relationship between cell proliferation and cell adhesion to a solid surface or another cell is poorly understood. It is known that anchorage can permit exit from the Go phase of the cell cycle, similar to the action of growth factors (Folleman, 1978; O'Neill, 1986). The expression of adhesion molecules is regulated in the process of initiating cell differentiation, maintaining the differentiated state of already differentiated cells and expressing specialized functions (Greenberger, 1991). Two remarkable features of cell adhesion include the fact that (1) the same ligands and receptors can regulate adhesion during such unrelated events as inflammation, the immune response, or hemopoiesis, (2) cell-ECM or cell-cell interactions by themselves regulate cell proliferation and differentiation.

The ECM produced by bone marrow stromal cells is composed of a variety of substances (Verfaillie, 1994). In addition to their role in facilitating cell-cell and cell-ECM adherent interactions, they also bind and present humoral hemopoietic regulators to the hemopoietic cells (Gordon, 1987). The ECM-binding growth factors, often have altered potency, increased stability and modified diffusion properties, and act on target cells more selectively than do diffusible growth factors alone (Flaumenhaft, 1991). In addition, certain proteins that act as cell adhesion substrata now appear to be important mitogens for immune and hemopoietic cells (Springer, 1990; Long, 1992). The activity that contributes to proliferation in established cell lines appears to be contained in parts of the molecule rich in EGF-like repeats (Mann, 1989). In cooperation or synergy with ILs and CSFs, such EGF-like domains in matrix proteins might have more general importance as localized signals for growth and differentiation (Pierce, 1988). Analysis of these molecules has revealed a mitogenic capability, either dependent on or independent of their adhesion domains, suggesting that their mitogenic effects are

mediated and regulated by specific receptors or by means of conformational changes of the same adhesion receptors (Engel, 1991; Hynes, 1992). It is known that receptors for ILs and CSFs are few, 50-5000 per cell, while receptors for mitogenic ECM proteins are 20,000-30,000 per cell on lymphoma derived cell lines (Levesque, 1990; Cohen, 1986). Usually, most of these adhesion proteins can be physiologically cleaved into small bioactive fragments with a molecular weight closer to that of hormones, growth factors, inhibitors, or chemotactic peptides.

3.3.2. Adhesion molecules of the hemopoietic system

During self-renewal and differentiation, hemopoietic cells manifest (directly or indirectly) changing patterns of adhesive interactions with other cellular or ECM components. The molecular basis of cell adhesion of immature hemopoietic cells to the BM stroma is complex. A wide variety of adhesion molecules (CAMs and ECM) involved in this process regulate cell-ECM and cell-cell interactions and play a role in proliferation, activation, release, and homing of the hemopoietic cells at all stages of maturation (Albelda, 1990; Tavassoli, 1990). These molecules can be grouped in several classes according to their structural homology or other structural/functional similarity (Williams, 1988; Albelda, 1990). These classes include the integrins, the cartilage link protein family (selectins and lectins), the immunoglobulin superfamily and cell surface proteoglycans. The expression of surface molecules related to adhesion reactions between hemopoietic and stromal cells are summarized in Table 4.

Table 4. ECM and surface molecules of bone marrow stromal cells related to progenitor homing and proliferation

ECM or surface molecule	Receptor	Hemopoietic cell bound
Fibronectin (Fn)	VLA-3, VLA-4, VLA-5	Committed progenitors
Hemonectin (Hn)	?	Multiple stem cells, CFU-G
Vetronectin (Vn) & vWF	αIIbβ3, αVβ3	Neutrophils, lymphocytes
Mannosyl, galactosyl residues	Lectins	Hemopoietic stem cells
Heparan, Heparan sulphate	N-CAM	Multiple cell types
Vascular addressin	H-CAM/CD44	Lymphocytes
(MEL-14, LEU-8/TQ1)	L-selectin	Lymphocytes
GMP-140	E-selectin	Neutrophils
Tetrapeptide	?	Hemopoietic cells
Low density of lipoprotein R	?	GM-CSF receptor
ic3b, Fb, TPS	Leu-M5	Monoytes,activated granulocyte, hairy cells,T cells
N-CAM, PECAM-1	N-CAM, PECAM-1	Lymphocytes,Neutrophils, $CD34^+$ cells
MEL-1, MAC-1	I-CAM-1	Neutrophils, T,B,Megakaryocytes
I-CAM-1, I-CAM-2, I-CAM-3	LFA-1, MAC-1 CR3, CD43	T.B. Leukocytes BFU-E,CFU-GEMm,CFU-M
V-CAM-1	VLA-4, VLA-7	Monocytes, Progenitors
BL-CAM	CD45	Leukocytes, Progenitors
Membrane-anchored M-CSF	M-CSF receptor	Progenitors
Membrane-anchored KL	c-kit	M-CSF receptor positive cells Mast

ICAM-1 (CD54) is a cell surface 90 kDa glycoprotein originally identified by a mAb that blocks homotypic aggregation of phorbol ester-stimulated leukocytes (Dustin, 1986; Horley, 1989). This molecule is expressed on a wide variety of cells including: (1) hemopoietic cells such as tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal centre dendritic cells in tonsils, lymph nodes, and Peyer's patches (Arkin, 1991), and (2) non-hemopoietic cells. ICAM-2 is constitutively expressed by vascular endothelial cells, thymic epithelial cells, certain other epithelial cells (Staunton, 1989). ICAM-1 is expressed at low levels on marrow stromal cells and resting endothelium; however, it is dramatically upregulated by IL-1, IFN-γ and TNF-α (Dustin, 1986; Pober,

1987). ICAM-1 mediates adherent interactions between T, B, promonocytic cells, and endothelial cells through LFA-1 and Mac-1 (Makgoba, 1988; Smith, 1989; Diamond, 1991; De Fougerolles, 1991).

VCAM-1 is a 110 kDa glycoprotein expressed on vascular endothelial cells after treatment with inflammatory mediators (Rice, 1989; Elices, 1990). It also is expressed on non-vascular cell types such as tissue macrophages, dendritic cells (Freedman, 1991; Rice, 1991), and marrow fibroblastoid cells (130 kDa) (Simmons, 1992). VCAM-1/VLA-4 ($\alpha_4\beta_1$) and VCAM-1/VLA-5 ($\alpha_5\beta_1$) are the ligand-receptor pairs involved in cell-cell interactions, especially in the maintenance of murine lymphopoiesis *in vitro* (Miyake, 1992; Elices, 1990). The VCAM-1/VLA-4 interaction is only partially responsible for adherence between BFU-E, CFU-GM and bone marrow stromal cells, however, (Simmons, 1992) because anti-VCAM-1 antibody alone or in combination with the EIDVPST peptide can not completely abrogate the binding. The expression of VCAM-1 on marrow fibroblastoid cells was upregulated by IL-1, IL-4 and TNF-α (Simmons, 1992), suggesting that this adhesion mechanism may be important in both normal (steady state and stimulated) hemopoiesis such as occurs in inflammation or after BMT. Conversely, TGF-β down-regulates VCAM-1 expression on marrow stromal cells (Dittel, 1993).

NCAM is a cell surface glycoprotein, 23-225 kDa, consisting of at least three isoforms (Murray, 1986). It is expressed on stromal cells in LTBMC that support lymphopoiesis (Thomas, 1988; Gordon, 1990). It is also expressed on mature T lymphocytes and NK cells (Lanier, 1986, 1989). Generally, hemopoietic progenitors do not express NCAM, but abnormal expression had been noted in some malignancies (e.g. chronic myelogenous leukemia and lymphomas) (Miller, 1991; Fern, 1992). In non-hemopoietic system, it is widely distributed in the central and peripheral nervous systems (Rutishauser, 1988; Cunningham, 1987) where it influences a wide variety of diverse cellular events (Rutishauser, 1988). It aggregates at sites of homologous and heterologous cell-cell adhesion (Bloch, 1992; Clark, 1992), but does not accumulate at sites of cell adhesion to the substrate (Pizzey, 1989).

Fibronectin (FN), abundant in normal bone marrow, is a principal component of the ECM produced by marrow stromal cells, and has been shown to support cell migration *in vitro* and *in vivo* (Rouslahti, 1988). The VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) complex are widely distributed and functions as a receptor for FN during erythroid differentiation (Wayner, 1988; Rosenblatt, 1991; Coulombel, 1992). FN also can promote the adhesion of murine CFU-S, CFU-E, BFU-E, LTCIC, and erythroleukemic MEL cells but not CFU-GM (Patel, 1984; Tsai, 1987; Vuillet-Gangler, 1990; Williams, 1991; Verfaillie, 1991; Coulombel, 1992). These interactions are mediated (at least in part) through the RGD, HBD, and CS-1 domains of FN and $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins (Verfaillie, 1991; Williams, 1991; Coulombel, 1992).

Hemonectin (HN) is a 60 kDa, tissue-specific adhesion protein (Campbell, 1987). It is restricted to the bone marrow, and initially was reported not to be present in the spleen, mammary gland, or kidney. It can promote the adhesion of murine granulocytic cells as well as CFU-GM and HL-60 (Campbell, 1992).

Integrins. The integrin family and other transmembrane proteins are considered to be the predominant adhesion receptors by which cells attach to ECM and/or to other cells (Tuckwell, 1993). The integrin family consists of at least 21 distinct heterodimers formed by associations between one of 13 α and 7 β subunits (Venstrom, 1993). Most cells express several integrins, and most integrins are expressed on wide variety of cells. Also, individual ligands are recognized by more than one of the integrins, and individual integrins can bind to more than one ligand (Soligo, 1990; Hynes, 1992). Many cells simultaneously display multiple integrins with overlapping binding specificity, but the biological significance for this redundancy is still unclear. It has been suggested that many integrins bind to ECM proteins that mediate cell-ECM interactions, while others bind to cell membrane proteins (counter receptors) that mediate cell-cell adhesion (Miyatani, 1989; Papayamopoulou, 1992).

Other molecules that may or may not be relevant to *in vivo* adhesion reactions include the following: (1) A 37 kDa protein expressed on murine marrow stromal cells, which is a ligand protein for a 110 kDa membrane protein of murine myeloid progenitor

cells that may mediate adherent interactions in homing of hemopoitic progenitor cells to the bone marrow (Shiota, 1993). (2) CD44 (H-CAM), expressed by immature erythroid cells, granulocytic progenitors, and lymphoid cells, might be involved in the release of hemopoietic cells to the circulation (Lewinsohn, 1990). (3) Membrane-anchored forms of growth factors and their receptors may also mediate cell-cell adherent interactions, as well as signal transduction. TGF-α, SCF or kl, and M-CSF can mediate cell-cell adhesions through binding to the EGF receptor, *c-kit* protein, and M-CSF receptor, respectively (Anklesaria, 1990; Flanagan, 1991; Uemura, 1993). (4) A growing number of plasma membrane molecules of unknown function, such as Thy-1, also may be involved in the adherent interaction between hemopoietic and stromal cells (He, 1991).

3.3.3. The ST3 molecule

The ST3 antigen was first reported as a phenotypic marker of rat marrow fibroblastoid cells (Sullivan, 1989). It was identified by an IgM monoclonal antibody produced by immunizing mice with cultured fibroblastoid cells of rat bone marrow (Sullivan, 1989). It is expressed on the predominant population (>99%) of cultured rat bone marrow fibroblastoid cells, half of splenic fibroblastoid cells, a subpopulation of neurons, cortical thymocytes, stromal cells (nurse cells) of the thymus, uncharacterized cells of the glomerular mesangium, and on only a minority of fibroblastoid cells derived from selected non-hemopoietic tissues (e.g. lung) (Sullivan, 1989; Sharma, 1991). It is also expressed on fibroblastoid cell precursors (CFU-F), but not CFU-C as shown by an antibody-dependent complement-mediated lysis assay (Sullivan, 1989). In addition, it is expressed on approximately 7% of unfractioned bone marrow cells in the small cell ("lymphoid") window when examined by flow cytometry (Sullivan, 1989). Except for differences in the brain, such a distribution is reminiscent of the Thy-1 antigen (Sharma, 1991). Although the physiologic function of the ST3 molecule is not yet known, its cellular and tissue distribution raises the possibility that it might be a cell interaction molecule of the marrow, thymus, and brain.

3.4. Spatial distribution of the hemopoietic microenvironment

Hemopoietic cells are not distributed at random within the marrow (Schofield, 1978; Lambertsen, 1984; Lord, 1990). The HM is thought to regulate hemopoiesis by providing specific combinations of cytokines and by establishing direct interaction with hemopoietic cells. As stem cells differentiate into more mature hemopoietic cells, they may move from one type of niche to another. This issue was supported by the following observations, (1) in murine bone marrow, a kinetic gradient of DNA replication (tritiated thymidine labelling) extends from the endosteal surface toward the central region, the greatest concentration of CFU-S is located nearest the bony surfaces, and most CFU-C are localized toward the central region (Lord, 1975; Gong, 1978; Mason, 1989), (2) the distribution of CFU-F coincides with the more primitive stem cells (Yang, 1984), (3) hemopoietic or stromal cells bind to ECM in a lineage or stage specific manner (Miyake, 1991; Williams, 1991), and (4) different hemopoietic regulators are produced or delivered by different stroma (Lord, 1990; Moore, 1991). Together, these lines of evidence indicate that the HM plays a critical role in regulation of lineage and stage specific differentiation of hemopoietic cells.

3.5. Traffic of hemopoietic cells between the bone marrow and peripheral circulation

Normal hemopoietic progenitors home to the bone marrow and spleen after intravenous infusion. This issue was derived from homing studies by transplantation of stem cells (unlabelled, gene-marked or fluorescence-labelled) to myeloablated or non-myeloablated recipients (Tavassoli, 1990; Heslop, 1995; Hendrikx, 1996). The identified homing receptors include calcium-dependent lectins (Aizawa, 1988; Matsuoka, 1989; Stoolman, 1989; Hardy, 1991), CAMs which are related to the LEC-CAM or selectin family (Springer, 1990), and known growth factor receptors, such as *c-kit* ligand (Flanagan, 1991). Evidence has shown that the mechanism of homing of erythroid progenitors might differ from that of CFU-S and CFU-GM. As indicated by experiments with synthetic probes, including galactosyl- and mannosyl-BSA, both CFU-S and CFU-GM could be selectively agglutinated from whole bone marrow. Both residues appear to be necessary for such binding, because competitive inhibition with one abolishes the

binding of the other (Aizawa, 1988). However, BFU-E and CFU-E were selectively agglutinated by mannosyl-BSA and fucosyl-BSA (Matsuoka, 1989; Konno, 1990). In addition, homing of hemopoietic progenitor cells to the spleen was mediated by a different mechanism (Konno, 1990). It appears that the ligand for the homing protein contains a yet unknown configuration of membrane carbohydrates. Treatment of stromal cells with neuraminidase followed by galactosidase and mannosidase reduced or nearly abolished the homing of hemopoietic progenitor cells to stromal cells (Matsuoka, 1989).

Normally, in adult mammals, hemopoiesis is limited to the bone marrow, and only mature blood cells are released into the peripheral circulation. Whether hemopoietic cells are released or not is considerably determined by the nature of their binding interactions within the stroma of HM. During the process of lineage programming, the expression of adhesion molecules or their receptors on hemopoietic progenitors is progressively changed and/or eventually lost (Williams, 1991; Liesveld, 1991; Koenigsmann, 1992).

4. FUNCTIONAL CHARACTERIZATION OF THE HM IN DIFFERENT BLOOD FORMING ORGANS - EMBRYO, FETUS, AND ADULT.

In mammals, hemopoiesis begins in the extra-embryonic mesoblastic tissue (yolk sac) where mesenchymal precursors differentiate into endothelial and hemopoietic cells (Pardanaud, 1989). At midgestation, it takes place at intra-embryonic sites (liver and spleen), where again it is closely associated with the mesenchyme of these organs (Magliaccio, 1986). Shortly before birth and thereafter, it settles at the medullary site of the bone, where stromal cells of the HM definitively associate with developing hemopoietic cells (Hann, 1983; Migliaccio, 1986; Christensen, 1986). Yet the mechanisms controlling how hemopoiesis shifts from one organ to the other during fetal life, and switches from a fetal to adult phenotype is not fully understood.

4.1. Yolk sac hemopoiesis

In some vertebrates, yolk sac hemopoiesis occurs in distinct foci called blood islands, which include a population of primitive stem cells that can give rise to committed progenitor cells, primitive and definitive red blood cells, granulocytes, and platelets (Tavassoli, 1991). However, in most species during embryogenesis (including mice and humans), the environment of the yolk sac is restrictive in its inductive potential, and differentiation of primitive stem cells is restricted to large nucleated red blood cells containing embryonic hemoglobin and primitive macrophages (Moore, 1970). Yolk sac HSCs are capable of forming multilineage hemopoietic colonies both *in vitro* (Schmitt, 1991; Chen, 1992; Keller, 1993) and in the spleen of an irradiated adult recipient (Moore, 1970; Perah, 1976; Medvinsky, 1993). There is controversy about the role of mammalian yolk sac blood islands as the unique source of stem cells for definitive hemopoiesis during embryogenesis. The HM of the yolk sac is composed of endothelial cells lining the blood islands, and the hemopoietic cells develop intravascularly, along adjacent yolk sac mesoderm and visceral endodermal cells (Tavassoli, 1991). Two epithelial-like cell lines derived from the endoderm and mesoderm, respectively, support *in vitro* survival of hemopoietic cells to a greater extent than similarly established adult bone marrow stromal cells (Yoder, 1994). They also influence the differentiation of hemopoietic precursors in a manner analogous to the yolk sac environment *in situ*; they support less neutrophil but greater macrophage differentiation from the 5-FU resistant hemopoietic cells than adult kidney or bone marrow derived stromal cell lines (Yoder, 1994). These results suggest that immortalized cell lines derived from the yolk sac recapitulate some of the microenvironmental influences that exist in normal yolk sac blood islands.

4.2. Transient hemopoiesis at extramedullary sites

During development, transient hemopoiesis occurs at different fetal organs/tissues; the most important of which are the liver and spleen. The beginning of definitive liver hemopoiesis is accompanied by wide dissemination of CFU-S in the embryonic tissues. The spleen colonies arising from fetal liver are similar to those derived from aorta gonad

mesonephrons (AGM) (Medvinsky, 1996). Hemopoiesis in these organs is also predominantly erythropoietic, and lymphopoiesis, myelopoiesis, and megakaryocyte production is less prominent (Timens, 1990). This is probably due to factors of the local environment. For example, a significant amount of erythropoietin (EPO) can be produced by fetal liver stroma (Slater-Cortenbch, 1987).

4.3. Hemopoiesis in the bone marrow

During evolution, hemopoietic bone marrow first appears in the frog. Except for ganoid fishes, lower vertebrates do not produce hemopoietic bone marrow (Tavassoli, 1991). In mammals, the bone marrow is of mesenchymal origin (Chevallier, 1977), and appears after a recognized bone has formed (Tavassoli, 1991). Bone marrow is the major hemopoietic organ which develops shortly before birth and thereafter. The histogenesis and homeostasis of bone and bone marrow is rarely disassociated. Yet, certain experimental data suggest that bone may not always be required for the development of hemopoiesis (Sadovnikova, 1991). The features of hemopoiesis in adult mammals are summarized as follows: (1) it is limited to the bone marrow, where it is the only organ in which myelopoiesis, erythropoiesis and lymphopoiesis proceed simultaneously, and (2) it occurs in the extravascular space separated by a marrow-blood barrier. The spatially organized HM of the bone marrow provides an optimal niche for hemopoiesis which is different from that occurring in other embryonic tissues. How the environmental factors of the bone marrow influence the HSC expansion/maintenance, lineage selection or programming, and maturation has been not elucidated.

4.4. Origin of hemopoietic stem cells

The existence of a common progenitor (hemangioblast) for hemopoietic cells and vascular endothelium has long been considered (Romanoff, 1960; Islam, 1992), although definitive evidence in favour of existence of this cell is lacking. Mice lacking TGF-β_1 exhibit variable defects of both blood and vasculature (Dickson, 1995). Also, in mice null for flk-1 (a vascular endothelial growth factor receptor expressed on endothelial cells and their embryonic precursors), deficient yolk sac blood islands, hemopoietic progenitors

and organized blood vessels will occur (Yamaguchi, 1993; Shalaby, 1995). Whether the hemopoietic deficiency is due to a lack of flk-1 expression in the hemangioblast or a primary defect in vasculogenesis is uncertain. Bidirectional transplantations have indicated that the two lineages have already segregated by day 3 of embryogenesis in the chick (Pardanaud, 1989). These two lineages of differentiation may occur together, but they are not necessarily associated. Capillary endothelium can develop in the absence of hemopoiesis, and hemopoietic foci can be seen in the absence of endothelium (Pardanaud, 1989; Dieterlen-Lievre, 1992).

4.5. Characterization of hemopoiesis in rats

In the rat, bone marrow comprises 3% of the adult body weight (Schermer, 1967). Most of the bony cavities consist of red marrow except for the distal two-thirds of the tail where there is yellow marrow (Hardly, 1967). The nucleated cell count ranges from 3.4 x 10^6/ml to 22 x 10^6/ml with minimal sex difference (Hulse, 1964). A significant reduction in cell number (2.68 x 10^6/ml to 3.45 x 10^6/ml) occurs between 30 and 40 days of age. In fetal rats, the major site of megakaryopoiesis is the liver, then the spleen assumes this function until a few days after birth, and thereafter, megakaryopoiesis takes place in the bone marrow. Approximately 40 days after birth, the bone marrow becomes the major site of platelet production (Marien, 1968). The hemopoietic progenitors can be detected in rat to rat and rat to mouse spleen colony assays (SCA) (Martens, 1986). Also in the rat to mouse assay, the colony numbers varied consecutively if compared to a mouse to mouse assay (Martens, 1986), raising questions about the compatibility of inter-species stromal-progenitor cell interactions.

5. LEUKEMIA AND THE HEMOPOIETIC MICROENVIRONMENT.

Myeloid leukemia is caused by transformation of an early myeloid progenitor cell or stem cell, and is characterized by an abnormal expansion of the malignant cells which leave the bone marrow prematurely and circulate in the peripheral blood. Most animal systems have failed to take into account the probable origin of leukemia and their natural

routes of metastatic spread. Abnormal proliferation and trafficking of leukemic cells might be influenced by the microenvironmental factors of the bone marrow (Denkers, 1992; Greenberger, 1992; Gabius, 1994, Liesveld, 1994).

5.1. *Leukemia survives and acquires a growth advantage in the bone marrow*

Leukemic cells exhibit a wide range of abnormalities in proliferation and differentiation, including (1) various degrees of growth factor dependence, (2) production of their own growth factor(s) (autocrine), (3) response to normal growth factors from other leukemic cells (paracrine), (4) expression of antigens or CAMs found in the normal nervous system or in embryo mesenchyme, but not present on normal hemopoietic progenitors and precursors, and (5) production of CAMs or adhesion receptors with altered number or affinity (Sutherland, 1988; Denkers, 1992; Sachs, 1993; Liesveld, 1994).

The most preferred organ for the growth of myeloid leukemic cells is the bone marrow (Hagenbeek, 1977a; Kamenov, 1984; Martens, 1987). *In vitro*, the leukemic cells exhibited heterotypic interactions with bone marrow stromal cells or their ECM (Gordon, 1987; Liesveld, 1991). These interactions may be required for leukemic cells to proliferate, and escape from natural apoptosis or death from chemotherapy (Manabe, 1992; Weber, 1994). Leukemic CD34+ progenitor express the ICAM-1, $\alpha_4\beta_1$, $\alpha_5\beta_1$, CD44, and sugar receptors that are thought to be important in the adhesion of normal hematopoietic progenitors to bone marrow stroma (Maio, 1990, Liesveld, 1994, Gabius, 1994). The adherent interaction of leukemic cells with the stroma might be determined by the expression of various receptor/ligand systems at a defined functional state. Expression of a non-functional receptor/ligand will result in impaired adhesion (Verfaillie, 1994). Such adherent interaction appears to be an important mechanism whereby leukemia cells are lodged in the bone marrow. The existence of growth factors which are secreted by adherent stromal cells and which strongly stimulate the growth of leukemic cells has been experimentally demonstrated (Greenberg, 1988; Greenberger, 1992). The cytokine receptors expressed by leukemic cells might be related to their intrinsic biological properties, as well as their reaction to the host's response to their

presence (Zola, 1994).

5.2. *Release and homing of leukemic cells*

The organ specific homing/metastasis is at least partially mediated through carbohydrate determinants which are recognized by their receptors. These receptors may be part of a larger family of recognition molecules which mediate normal cell-cell interaction (Glinsky, 1993). The expressed adhesion molecules and the homing behaviour of the leukemic cells are different from normal hemopoietic cells (Denkers, 1992; Verfaillie, 1994). In AML (acute myeloblastic leukemia), the release of leukemic cells from the bone marrow into the peripheral blood might be regulated by the degree of variation in expression of cell surface adhesion molecules on both stromal and leukemic cells, or to changes in receptor affinity (Denkers, 1993).

6. *IN VIVO MODELS TO STUDY THE DEVELOPMENT OF HEMOPOIETIC CELLS WITHIN THE MICROENVIRONMENT*

Establishment of the hemopoietic system *in vivo* requires expansion/maintenance, growth, differentiation, and maturation of hemopoietic cells in response to growth signals provided either locally or systemically. In an intact animal, hemopoiesis is dynamically regulated by direct contacts between stromal and hemopoietic cells, and by soluble factors. However, the production or action of a single regulator may be influenced by other factors acting locally or systemically through complex networks. Therefore, any *in vitro* hemopoietic system imperfectly represents hemopoiesis *in vivo*.

6.1. *Severe combined immunodeficient (SCID) mice and chimeric animals*

Stem cells not only have an extensive capacity to proliferate and differentiate into multiple blood cells lineages, but also are able to self-renew, as confirmed by many studies showing long-term reconstitution of hemopoiesis after BMT. By taking advantage of the immunologically permissive SCID mouse, two *in vivo* models were established

using human/mouse (McCune, 1988; Lapidot, 1992) and human/sheep chimeras (Srour, 1993) to characterize normal or leukemic human stem cells. Multilineage hemopoiesis was maintained in SCID mice for at least 14 weeks, suggesting that early stage hemopoietic cells had engrafted and were maintained in the murine HM. However, the injection of human HSCs into SCID animals has not been sufficient to achieve long-term engraftment within the marrow of these recipients, although much higher levels of human hemopoiesis ($> 10\%$) occurs in mice treated with growth factors than in those which were not ($< 1\%$) (McCune, 1988, Lapidot, 1992, Kyoizumi, 1993). When hemopoietic progenitors ($CD34^+$) were co-transplanted with engineered primary stromal cells expressing IL-3, the multilineage progenitors were recovered from the marrow of recipients for up to 9 months (Nolta, 1994). However, growth factors were not required to establish high levels of human hemopoiesis in SCID mice by transplantation of cord blood cells (Vormoor, 1994). In contrast to the SCID mouse, the transplantation of human HSC into preimmune fetal sheep (human/sheep chimera), shows that it requires a larger number of cells or co-transplantation of human tissues. A full lineage of hemopoiesis can be sustained in the human/sheep chimera up to 2 years post transplantation (Srour, 1993).

6.2. Bone marrow transplantation

Allogenic or autologous BMT is another model which can be used to study the HM regulating hemopoiesis *in vivo*. The major problem for the BMT is the source of convenient transplantable HSCs and long-term survival in an immunopermissive recipient. Also the HM of the recipient may be influenced by the disease condition (Bhatia, 1995) or by the preparatory regimen for the BMT, such as irradiation or chemotherapy drugs (Verfaillie, 1990; Shirota, 1990). The source of hemopoietic progenitor cells enabling transplantation can be from marrow, peripheral blood, umbilical cords, and placenta (McGlave, 1991; Gale, 1992). Peripheral blood-derived stem cells provide many advantages in therapeutic indications, but the frequency of convenient stem cells or progenitor cells in the peripheral blood is still less (Gale, 1992), although their concentration can be increased by application of cytotoxic agents (e.g.,

cyclophosphamide) or hemopoietic growth regulators (Neben, 1993; Grzegorzewski, 1994; Yan, 1994). *Ex vivo* expansion of stem and progenitor cells may become feasible using combinations of cytokines but possibly at the cost of diminished quality of the earliest cells.

7. *BONE MARROW INDUCED BY BONE MORPHOGENETIC PROTEIN*

Because of the limitations of the models mentioned above, the need exists for other models with which to study mechanisms of HM regulation *in vivo*. Because the bone/bone marrow repairs itself in a manner similar to the way it forms in an embryo, the possibility may exist for inducing the formation of a *de novo* bone/bone marrow in post-fetal mammals. A model exists in which ectopic bone/marrow is induced by bone matrix powder or purified BMPs (Reddi, 1980; Wozney, 1988).

7.1. General characteristics of BMP

The study of BMP began with the demonstration that bony nodules formed where decalcified bone matrix had been implanted at an extramedullary site (Urist, 1965). Extraction and purification of the substance revealed that the active constituents using *in vivo* assays were acidic polypeptides with a molecular weight of about 18.5 ~ 30 kDa (Urist, 1984; Sampath, 1987; Wang, 1988). The full-length cDNAs encoding three polypeptides (STPAQDVSR, NQEAL, and XANVXEN) have now been cloned and the gene products designated as BMP-1 through BMP-8 (Wozney, 1988; Celeste, 1990). The derived amino acid sequence of BMP-1 appears to be unrelated to other known growth factors, but the seven other BMPs (BMP-2 to BMP-8) are homologous and contain gene sequences related to other members of the TGF-β family (Centrella, 1994; Kingsley, 1994). Homologies of TGF-β family members and the the ability to induce bone are summarized in Table 5.

Table 5. Homologies of TGF-β family members and the capacity to induce bone.

Member	% identity*	Bone induction#
Drosophila dpp	100	+
BMP-2	74	+
BMP-4	76	+
BMP-3 (osteogenin)	43/40 (dpp/60A)	+
Drosophila 60A	100	+
BMP-5	72	+
BMP-6	71	+
BMP-7	69	+
BMP-8	65	?
Xenopus Vg-1	50	?

* Amino acid sequence identity in the carboxyl-terminal mature domain. # Bioassay for *in vivo* cartilage and bone induction: +, positive; ?, unknown.

7.2. Osteoinductivity of the BMP

The osteogenic activity of purified or recombinant BMP is tested by implanting it at an extraskeletal site (Table 5) (Urist, 1965; Urist, 1984; Reddi, 1980; Wozney, 1988), where an ossicle forms after a complex cellular cascade involving the migration, proliferation, condensation, and differentiation of undefined mesenchymal cells (Wozney, 1988). The incidence of bone formation and the mass of the induced bone is influenced by many biological and physiological factors that include the following: (1) the species of the recipient [the osteoinductivity of BMP implants being high in rodents] (Urist, 1969); (2) the site of implant (Wlodarski, 1986); (3) the age of recipient [older animals being less responsive] (Irving, 1981); (4) non-specific and specific immune reactions of the recipient [the osteogenic activity is almost totally lost if impure fractions are implanted into untreated normal rats, whereas high osteogenic activity is obtained with rats immunosuppressed by cyclosporin A] (Aldinger, 1991); (5) the components of the implant [use of an inactive allogenic bone matrix as a biological BMP carrier can correct the rapid absorption and inactivation seen when pure or recombinant BMP is implanted] (Reddi, 1994); and (6) the systemic hormonal status of the recipient [formation of the

ectopic endochondral bone was influenced by the growth hormone, thyroid hormone, and others] (Weiss, 1979; Reddi, 1985; Hashimoto, 1991). In the same assay system, single application of TGF-βs, activin-A, PDGF, hEGF, FGF, BDGF, SGF, insulin, IL-1, CDGF, bone growth hormone, and other peptide factors, does not produce activity in the ectopic bone formation assay, but each factor synergistically enhances BMP activity (Bentz, 1991; Ogawa, 1992).

Attempts to dissect the mechanisms *in vitro* have led to the following observations. The elements responding to the BMPs are considered to be local mesenchymal cells (Lyons, 1990). The chondrogenesis is influenced not only by BMPs, but also by many other growth factors, mitogens, and cytokines (Kawamular, 1988). These immature mesenchymal cells can be differentiated into fibroblasts, reticulocytes, adipocytes or osteoblasts in response to BMPs by *in vitro* assay (Whyte, 1989; Asahina, 1996). BMPs can mimic or oppose the effects of TGF-βs in various bone and bone cell culture systems. For example, BMPs directly stimulate chondrocyte proteoglycan synthesis in cultures of bovine articular cartilage (Carrington, 1990). Although TGF-βs can stimulate or inhibit chondrocyte differentiation, depending on the developmental stage (Centrella, 1991), BMPs can reverse the inhibitory effect of TGF-βs and PDGFs on proteoglycan synthesis in more differentiated chondrocytic cells from chick limb buds (Chen, 1991, 1992). In addition, BMP-2 appears to inhibit the stimulatory effect of FGF-type 4 on mesenchymal cell proliferation in intact mouse limb bud cultures (Niswander, 1993). These findings are consistent with the idea that progressive local interactions among multiple members of the TGF-β family or others induce bone formation (Kemmel, 1987; Lyons, 1989b). It still is not clear whether these multiple members have different levels of importance in a hierarchy of events in normal bone growth, remodelling, and fracture repair. It is known that BMP-2a and BMP-3 contain an EGF-like domain, as well as an "A domain" with sequence homology to a known protease (Titani, 1987). The TGF-β_1 binding protein also contains EGF-like domains, leading to the suggestion that it might be a protease involved in the activation of TGF-β_1 (Miyazono, 1988). Collectively, all of these observations suggest that BMP might be a proteo-hormone-like

protein, since its effect is confined to the site of implantation, and induces cell proliferation and differentiation locally.

7.3. Tissue distribution and other possible activities of BMPs

The expression of BMP transcripts is not only restricted in the developing skeletal system (bone and cartilage), but also includes others sites (Lyons, 1989b, 1990; Rosen, 1989; Jones, 1991; Vainio, 1993, Makashima, 1994; Takeda, 1994). In nonskeletal tissues BMP-2 mRNAs are expressed at sites of interaction between epithelial and mesenchymal layers (Lynos, 1989b). BMP-3, BMP-4, and BMP-7 transcripts are primarily expressed in lung, neural fold, and kidney/brain, respectively (Lee, 1991, Ozkaynak, 1991, Graham, 1994). The temporally and spatially distinct patterns of BMP mRNA expression in different developing systems suggest that BMPs are also involved in inductive events unrelated to bone induction. For example, BMP-2b is a chemoattactant for peripheral blood monocytes and increases their TGF-β_1 mRNA levels *in vitro* (Cunningham, 1992). This effect may be important in the context of the inflammatory response that occurs in the early phase of fracture repair. Injection of BMP-4 into Xenopus embryos promotes formation of ventral/posterior mesoderm (Dale, 1992; Jones, 1992a), and misexpression in mice blocks proliferation of hair cells (Blessing, 1993). Also, evidence exists that it may be a signalling molecule which mediates apoptosis in the rhombencephalic neural crest (Graham, 1994). Thus, it seems likely that the role of BMPs in embryonic development, and in the morphogenesis of various tissue and organ systems, may be to counteract or limit the effects of other agents, independent of their potent effects on bone formation (Kingsley, 1994).

7.4. The transforming growth factor-β family of mesenchymal regulators

The transforming growth factor-β superfamily contains more than two dozen gene products closely related to TGF-β_1 (Kingsley, 1994). As a group, they appear to play an important role in the regulation of growth and development (Pelton, 1989; Thompson, 1989; Centrella, 1994). It is suggested that the coordinated expression of different members of this family is required to regulate the progression of specific cells

at specific times along specific differentiation pathways during mammalian development (Lyons, 1989b; Centrella, 1994).

TGF-β_1 is a 25 kDa homodimeric protein. It has been isolated from most types of cells, and has been proposed to be a key modulator of ECM homeostasis through its effect on expression of others genes, either stimulating or inhibiting cell proliferation and differentiation (Lund, 1987; Coffey, 1988; Russell, 1988). It enhances bone formation, presumably by accelerating normal cellular and biochemical mechanisms of repair (Noda, 1989; Marcelli, 1990; Beck, 1991; Richardson, 1993). Either alone or combined with basic fibroblast growth factor (bFGF) or TGF-β_2, it can induce the expression of mesoderm specific markers in isolated *Xenopus cap* cells (Rosa, 1988). Both TGF-β_1 and TGF-β_2 can induce fetal rat muscle mesenchymal cells to undergo differentiation and synthesize cartilage-specific macromolecules *in vitro* (Seyedin, 1987).

A large number of putative receptors for the TGF-β family have now been cloned (Kingsley, 1994). Although not all of these binding sites are expressed in all cells at all times (Attisano, 1994). Changes in TGF-β binding are observed during development, aging, and hormone stimulation in a variety of tissues (Centrella, 1994). BMP-2 and -4 have been shown to bind to putative serine/threonine kinase receptors with much higher affinity (Takeda, 1994; Harland, 1994). Also, BMP-2 can reduce TGF-β binding to type II receptors and betaglycans on fetal rat osteoblasts, without affecting type I receptors, and rapidly reduces betaglycan transcripts (Centrella, 1994). Many of the diverse cellular effects of TGF-β are considered to be the result of alterations in expression of the level of many different proteins including ECM, CAMs or their receptors, enzymes that control matrix deposition, cell cycle components, transcription factors and growth factors (Makashima, 1994).

The hemopoietic system is derived from ventral mesoderm. The members of TGF-β superfamily (activins, Vg-1, BMPs), FGF, and *noggin* have been shown as the mesoderm inducer in *Xenopus* (Slack, 1994). Interestingly, the BMP-4 and the BMP-2/4 receptor are essential for gastrulation and appropriate formation of mesoderm (Winnier, 1995). Furthermore, BMP-4 can induce hemopoietic activity in embryoid bodies formed from mouse embryonic stem cells and grown in chemically defined medium (Johnasson,

1995).

7.5. Molecular mechanisms possibly involved in bone/bone marrow regeneration

The sequence of marrow reconstruction in the adult might be viewed as a recapitulation of ontogeny (Fan, 1990; Reddi, 1994). It may be controlled by a similar genetic program (a group of genes) to that of histogenesis in both embryo and certain regenerating adults tissues. One family of genes, the "homeobox", has been identified in mice (Hart, 1987) and humans (Simeone, 1987), and are expressed in a lineage-specific manner (Gehring, 1987; Kongsuvan, 1988; Tickle, 1994). In mammals, 38 genes containing a homeobox sequence have been identified that are related to the Drosophila *Antp* gene (Simeone, 1991; Duboule, 1992). Genes belonging to the *hox-4* complex have distinct expression patterns during limb development (Dollé, 1989). They are first expressed in undifferentiated mesenchymal cells at the limb bud stage, and are subsequently restricted to precartilagenous blastemas and perichondrium at later stages (Dollé, 1989). A large number of homeobox genes also are expressed by blastema cells during limb regeneration (Gardiner, 1993), and appear to be involved in growth control and pattern formation. The BMPs can induce expression of homeobox genes in a dynamically changeable pattern (Iimura, 1994).

CHAPTER 2

THE PATTERN OF PROGRESSION OF RAT MYELOID LEUKEMIA (BNML) DIFFERS IN BONE MARROW AND SPLEEN

PREFACE

Clinical evidence indicates that homing of hemopoietic cells or metastasis of primary tumors is not random. Mechanisms to explain how microenvironmental factors of the host tissue influence hemopoiesis under normal or leukaemic conditions *in vivo* is not fully understood. By identifing molecules related either to homing or to the growth of leukemic cells might provide better understanding of the complicated *in vivo* situation, and could also point the way to new therapeutic strategies. Consistent with these concepts, as a starting point for the work contained within this thesis, I examined the relative capacity for leukemic cells to grow in the bone marrow and spleen using the rat myeloid leukemic cell line (BNML) as a model.

ABSTRACT

Leukemic cells arise and proliferate in the bone marrow, but the mechanisms by which progeny of the original clone invade other sites is not well understood. Rat myeloid leukemia cells (BNML) that had been labelled with a fluorescent dye (CMFDA) and also bore the lacZ marker gene were injected into recipient animals, and the subsequent pattern of growth observed. At 72 h, less than 0.3% of the cells were detected in the peripheral blood, bone marrow, or spleen. Irradiation 24 h prior to cell infusion resulted in a five-fold increase in marrow infiltration. After 18-21 days, when the marrow was almost completely replaced by leukemic cells, the spleen was minimally involved. Leukemic cells were not detected in the thymus at any time. In contrast, normal clonogenic myeloid cells were abundant in the spleen and depleted from the marrow. Conditioned media prepared from activated spleen cells, although stimulatory for the growth of normal myeloid precursors, completely inhibited the *in vitro* growth of BNML cells. This model may be used to study how tumour cells interact with the microenvironment of a target organ, and how the process is influenced by physical agents such as radiation.

ABBREVIATIONS: *BNML*, Brown Norway myeloid leukemia; *CFU*, colony-forming unit; *CMFDA*, 5-chloromethyl fluorescein diacetate; *FCS*, fetal calf serum; *SCCM*, spleen cell conditioned medium.

INTRODUCTION.

Human myeloid leukemia is thought to originate in the bone marrow, and appears not to infiltrate other organs to the same extent until late in the course of the disease. Because it usually arises by clonal expansion and evolution from a single cell, the fact that it disseminates to other intramedullary loci implies that the cells home to and find their most permissive microenvironment within the marrow. Being clinically asymptomatic, this phase of the disease is difficult to investigate in humans, and animal models may be useful.

Evidence supports the idea that homing of normal hemopoietic stem cells, as well as metastasis, is a non-random, organ-specific process that involves multiple factors, including expression of surface molecules on both the migrating cell and target organ (receptor-accepter hypothesis), and humoral factors of the local microenvironment (seed-soil hypothesis) [1][2][3][4][5][6][7]. The Brown Norway rat myeloid leukemia (BNML), extensively characterized by Hagenbeek and Martens [8][9], has been used to examine the process of leukemia cell localization. Studies with this model suggest three phases after intravenous injection: (1) initial localization of the infused cells; (2) recirculation and proliferation within the preferred organs; and (3) rapid generalized proliferation leading to death of the animal. Between each of these stages the leukemic cells may behave and interact differently with cells of the microenvironment of the host organ, and this process has not been well characterized. Here we describe experiments comparing the growth of BNML cells in the marrow and spleen in the early and intermediate phases after intravenous injection.

MATERIALS AND METHODS

Animals. Inbred male and female Brown Norway (BN) rats of age 10-12 weeks (purchased from Charles River Canada; St. Constant, Que) were housed in polycarbonate plastic cages and received standard laboratory chow and water *ad libitum*. For certain experiments they were subjected to total body irradiation from a cesium dual-source irradiator (Gammacell 40; Atomic Energy of Canada, Limited, Ottawa, Ontario) at a dose rate of 139.4/min and a total dose of 808.5 rad, and injected with leukemic cells within 24-48 h.

Manipulation of cells. The cell line LT12NL15, a subline of the BNML rat myeloid leukemia into which the lacZ gene had been stably inserted, was a generous gift of Drs. P.J. Hendrikx, A. Martens, and A. Hagenbeek (Erasmus University, Rotterdam, The Netherlands) [10]. It was maintained by *in vitro* passage in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

For experiments using dye labelled cells, 5-10x10^7 LT12NL15 cells were washed with RPMI medium and resuspended in medium containing 10-30 μM CMFDA (5-chloromethyl fluorescein diacetate, Molecular Probes, Inc, C-2925) and incubated at 37°C for 20-30 minutes. The cells were then pelleted by centrifugation, resuspended in dye-free medium, and then incubated for another 30 minutes before repeat washing and resuspension. A small fraction was immediately analyzed by flow cytometry (Epics Profile) at 490-520 nm to determine the labelling efficiency (>99%). For each set of experiments, the gates were set for forward and side scatter to include 99% of the BNML cells (the "BNML window"). An aliquot of 5.0x10^7 with a viability of >95% was injected through the tail vein, and after 72 h the rat was sacrificed by ether overdose, and the spleen and bone marrow were recovered for the preparation of a cell suspension for further analysis. For experiments examining later time points, 1.0x10^7 cells that had not been dye-labelled were injected similarly and examined after 20-21 days; an occasional animal became afflicted with hind limb paralysis by day 18, and was sacrificed at that time.

Cell suspensions were prepared from normal bone marrow by flushing the femoral shafts with cold medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cell suspensions of spleen and thymus were prepared by mincing the tissue in RPMI medium, and dispersing the aggregates through a fine mesh sieve. Blood samples from rats were withdrawn from the heart and placed into EDTA-containing "microtainer" tubes (Becton, Dickinson, Vacutainer). These cell preparations were centrifuged over a Ficoll-Hypaque barrier, nucleated cells were collected from the interface, washed three times with regular medium, and finally resuspended in the appropriate medium, depending on subsequent use.

Preparation of spleen cell conditioned medium (SCCM). This was as previously described [11][12]. Briefly, nucleated spleen cells ($1x10^6$/ml) were cultured in RPMI 1640 medium containing 10% FCS, 1% BSA, 2 mM L-glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml phytohemagglutinin (PHA-L Pure E.Y. Labs, San Mateo, CA). After 4-5 days the supernatant was harvested, centrifuged at 1200 rpm for 30 min, and stored at -20° C. The concentration of SCCM used was that which caused maximal stimulation of colony formation in preliminary assays (usually 5%).

Clonogenic cell assays. These methods were performed as described previously [11, 12]. Briefly, a cell suspension was prepared from normal bone marrow in RPMI 1640 medium containing 10% FCS, and mixed using a syringe with a 21 gauge needle. One ml of the culture system consisted of the following:

- Iscove's Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 30% FCS, 1% BSA;

- 5% spleen cell-conditioned medium (SCCM) for assay of CFU-G/M;

- $5x10^{-5}$ M 2-mercaptoethanol; 0.8% methyl cellulose (4000 centipoise; Fisher Scientific #M-352); and $1.5x10^5$/ml nucleated marrow cells.

The mixture was added to a 35 mm culture dish and incubated at 37°C in an atmosphere containing air/5% CO_2.

The CFU-BNML assay was similar to the normal CFU assay, except that neither

SCCM, nor erythropoietin was added. Both the CFU-G/M and CFU-BNML were enumerated on day 7 using an inverted microscope; a colony was defined to be >50 cells.

Assay for inhibitory activity. BNML cells were either distributed into 24-well culture dishes at $1x10^4$/well in RPMI 1640 medium containing additives noted above, or suspended into methyl cellulose at 150 cells/culture; varying concentrations of SCCM were then added. At times stated in the "Results", the cultures were harvested and the cells enumerated by flow cytometry (liquid cultures) or by colony count (semisolid cultures).

Visualization of lacZ-marked cells. Examination of β-galactosidase in cells was as described by Yan et al [10]. Briefly, the enzyme substrate solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Molecular Probes, Eugene, OR 974021, USA) was prepared immediately before use. Microscope slides on which cells had been applied by cytocentrifugation ($1.5\text{-}4.5x10^5$ suspension cells/slide; Cytospin 2, Shandon Southern Products, Ltd.) were fixed with 0.5% glutaraldehyde for 10 minutes and washed three times with PBS, and then incubated with the substrate solution overnight. The staining efficiency was 98-99%.

Colonies in methyl cellulose were stained *in situ* by adding 500 μl of the substrate solution on top of the methylcellulose layer in 35 mm cultured dishes 7 days after culture. After 4 h incubation at 37° C, a blue colour indicated the presence of enzyme-bearing cells (Fig 2A).

RESULTS

Early phase localization of leukemic cells and the effect of irradiation. Preliminanry experiments had shown that 10 min after IV injection of $5x10^7$ CMFDA-labelled BNML cells, a high proportion of leukemic cells were detected in the peripheral blood, lung, and spleen (fluorescent cells comprised 80-90%, 70-80%, and 40-50% respectively within the "BNML window"); fluorescent cells were not detected in the thymus or bone marrow. By 24 h, these percentages had decreased to 10-20, 5-10, 10-20, and remained negligible in the marrow. As shown in Fig. 1, by 72 h leukemic cells comprised less than 0.5% of the defined population in the blood, bone marrow, and spleen. However, application of sublethal irradiation 24-48 h prior to cell infusion resulted in a five-fold increase in leukemic cells in the bone marrow; a similar trend was noted in the spleen, which was not statistically significant.

Intermediate phase localization. After injection of $5x10^7$ BNML cells, animals will die in 23-25 days. Comparison of the cellular composition between bone marrow and spleen from animals sacrificed 21 days after injection is shown in Figs. 2B-E. At this time most of the cells in the bone marrow carry the lacZ gene (e.g., are leukemic), whereas only a minority of the spleen cells are leukemic. By inspection, most of the lacZ-positive cells in the spleen appear to be degenerating.

This morphological impression was confirmed by assay of clonogenic cells in the peripheral blood, spleen, and bone marrow (a representative BNML colony is illustrated in Fig. 2A). As shown in Fig. 3, very few clonogenic leukemic cells were detected in the spleen, whereas many were found in the bone marrow. Normal myeloid precursors, however, were depleted in the bone marrow and abundant in the spleen. Control cultures from rats that had not received a leukemic cell load did not reveal any CFU in the peripheral blood or spleen. Leukemic cells also were sought in the thymus, and only a single colony was found in assays on 14 rats (i.e., fewer than $1/10^4$), further supporting the notion that localization of BNML cells to hemopoietic organs is not random or simply a function of blood flow.

Effect of soluble factors derived from activated spleen cells. The apparent non-

permissive character of the spleen may depend on several mechanisms, including production of inhibitors. This possibility was investigated by culturing BNML cells *in vitro* in the presence of media obtained from normal rat spleen cell cultures ("SCCM"). Addition of SCCM resulted in a dose-dependent decrease in the growth of BNML cells (Fig. 4), in contrast to the usual stimulation that it produced with cultures of normal bone marrow myeloid precursors (102 $\pm$ 5 [SD]; N=30).

DISCUSSION

As originally described, the BN myeloid leukemia expressed promyelocytic features, was clonogenic *in vivo*, did not require prior irradiation to form colonies in the marrow and spleen, was maintained by *in vivo* passage, had an LD_{50} of 25 cells, and grew with predictable kinetics. Adaptations made to the original strain include establishment of the cells as a permanent line with a myeloblastic phenotype [13], and insertion of the lacZ marker (subline LT12NL15) that can be used to trace cell populations within an animal [10]. Using dye-labelled BNML cells and those in which the lacZ gene had been incorporated, we have confirmed and extended previous studies of Hagenbeek, Martens, and co-workers on the pattern of growth of leukemic cells in the bone marrow and spleen.

Based on observations of changes in organ weight and inspection of histological sections, Hagenbeek and coworkers reported that after injection of $1x10^7$ BNML cells the leukemic load in the spleen increased rapidly between days 12-16, but in the liver it increased only after d18-20. Prior to that time (d8) the frequency of leukemic cells in the spleen was $<0.04\%$ (assessed by *in vivo* animal mortality bioassay). Histological studies showed that after d18 the femoral marrow was completely replaced by leukemic cells. Between d8-18 large clusters developed in the splenic red pulp along. with increased megakaryocytes and red cell precursors, but it was not until 8-10 days premortem that the lymphoid follicles were overgrown by leukemia. This lag was attributed in part to a high fraction of cell loss (88%) over proliferation, the cause of which was not evident. Curiously, the thymus was not invaded at all, and in fact became atrophic. Examining normal CFU-C, they observed a rapid rise in the spleen, peaking at d18, in parallel to the loss of clonogenic progenitors from the bone marrow, and suggested that the stem cells were mobilized from the marrow and rerouted to the spleen as the leukemic cells occupied "niches" at the endosteal surface. Further kinetic studies implied, however, that the splenic microenvironment was permissive to proliferation and differentiation, but not to self-renewal of normal progenitors [8][[9][14][15]. All of these results argue that the metastatic localization and growth of BNML cells is not simply determined by

presentation of cells to the organ, but may involve more subtle interactions with the sinusoidal endothelium and the extravascular stromal complex.

The results we report here show that in the *early phase* (10 min to 24 h) after intravenous injection of 1-5x10^7 leukemic cells, a significant proportion remained in the spleen, but very few were found in the bone marrow. By 72 h, fewer than 0.3% of cells with flow cytometric properties similar to BNML (a small minority of the total cells of either organ) were detected. However, irradiation of the animal 24 h before infusion resulted in a five-fold increase in the leukemic cell infiltrate in the marrow. By 20 days, which precedes the pre-terminal phase of rapid proliferation in all affected organs, most cells of the marrow and only a few in the spleen were leukemic. This contrasts with the content of normal myeloid precursors, which were depleted in the marrow, but increased in the peripheral blood and spleen. Conceivably, this fact might account for the increase in spleen size in the intermediate phase, which could have been interpreted as being due to expansion of the leukemic cell mass if endogenously marked cells (e.g., lacZ) were not available to allow clear discrimination between the two cell populations [8, 10]. A possible factor contributing to this phenomenon may be the ability of spleen cells to release factors that stimulate normal myeloid precursors, but inhibited BNML cells.

Recent reports have focussed on the role of cytokines in the organ localization of myeloid precursors. For example, injection of IL-7 into mice for 7 days resulted in a 90% reduction in the CFU-C content of the bone marrow and in a 15-fold rise in frequency in the spleen caused by relocalization of the progenitors [16][17]. *In vitro*, it can synergize with SCF to enhance the formation of granulocyte colonies [18], or in the presence of TNF-α, promote macrophage colonies [19], as well as activate the tumoricidal potential of monocytes [20]. The presence of IL-7 on the subset of reticular stromal cells that concurrently express VCAM-1 suggests that it may contribute to hemopoietic regulation *in vivo* [21]. Although none of the results presented here purport that the behaviour of BNML cells is controlled by IL-7, it is reasonable to suspect that the interaction of stromal elements of the bone marrow and spleen with BNML could induce secondary effects. Secretory products of the leukemic cells could lead to further cytokine activation and subsequent modification of their homing pattern and capacity to

proliferate in a given microenvironment, as has been described during metastatic spread in other tumour models [22][23][24].

Factors released during these interactions could lead to stimulatory or inhibitory effects. Here we show that the same conditioned media from mitogen-activated normal rat spleen cells that stimulates normal CFU-G/M/GM [11] inhibited the *in vitro* growth of BNML cells in both clonogenic and liquid culture. This appears to be a different product, or combination of factors, from the one we have previously reported to be secreted by antibody-defined lung fibroblasts which inhibits the growth of normal rat CFU-C and clonogenic BNML cells, but not those growing in suspension cultures [25][26][27]. The fact that it reaches its half-maximal inhibition of BNML cells at a concentration ($\sim$ 4%v/v) lower than that which maximally stimulates normal CFU-C ($\sim$ 5%) argues that the effect could not be explained by non-specific toxicity. Further comparison of the two activities, however, must await biochemical characterization.

The observation that prior irradiation leads to augmentation of early phase entry of leukemic cells into the marrow might be explained by injury to the sinusoidal endothelium as described by Shirota and Tavassoli [28], or by subsequent modulation of adhesion molecules and/or cytokine expression [29]. Were the uptake of leukemic cells to be augmented by irradiation, therapeutic strategies that include reinfusion of unpurged autologous bone marrow could be compromised. Indeed, as the treatment of leukemic and other cancers focuses on the management of minimal residual disease, an understanding of the mechanisms controlling tumour cell localization in the early and intermediate stages will become more relevant.

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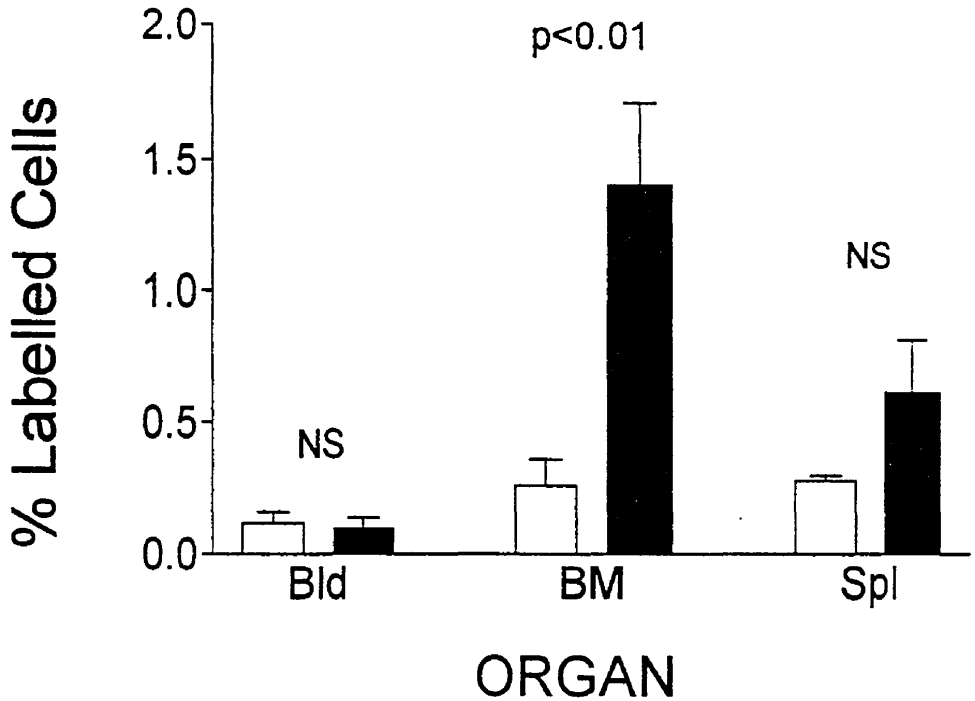
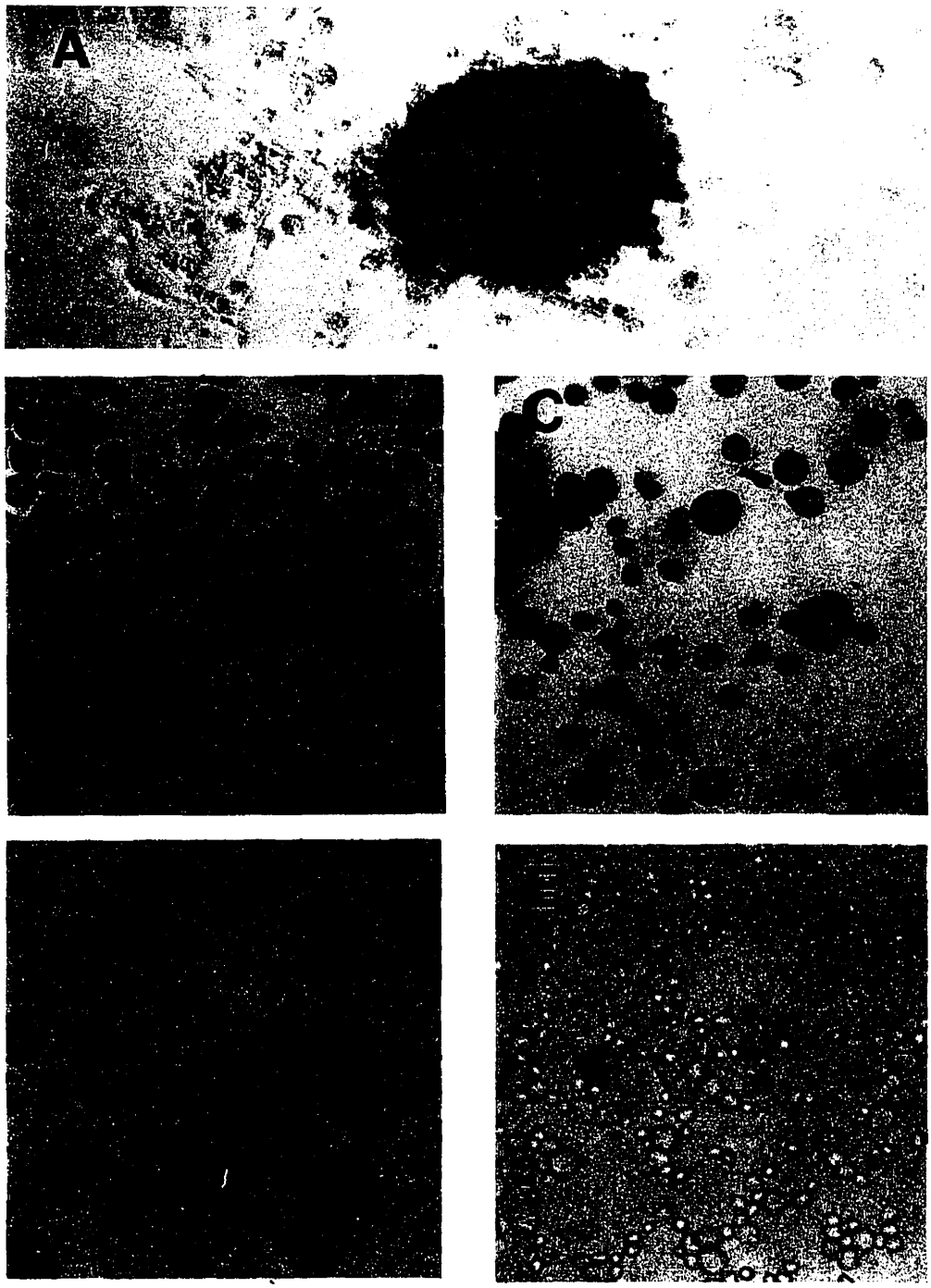
LEGEND TO FIGURE 1.

Effect of irradiation on the frequency of leukemic cells within various organs. Cell suspensions were prepared from peripheral blood (Bld), bone marrow (BM), and spleen (Spl), and the fluorescent cells (labelled by CMFDA) were assayed by flow cytometry, gating on a window previously found to contain the BNML cells (the "BNML window"). *Open bars*, animals not irradiated prior to injection of leukemic cells; *solid bars,* animals irradiated. Non-irradiated, mean ($\pm$ SEM) of 5 animals; irradiated, mean of 7 animals. Statistical significance between the values measured for leukemic cells in the non-irradiated vs. irradiated animals was tested by one-way ANOVA (Tukey-Kramer test).


% Labelled Cells
2.0
1.5
1.0
0.5
0.0
p<0.01
NS
NS
Bld
BM
Spl
ORGAN

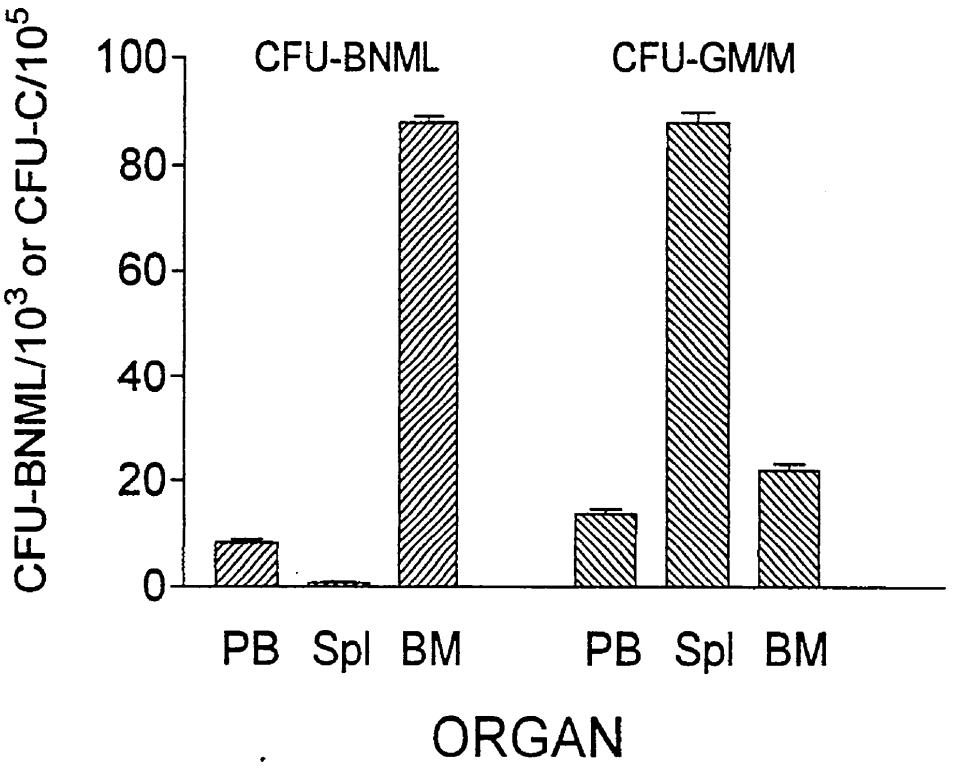
LEGEND TO FIGURE 2.

Photomicrographs of BNML cells from bone marrow and spleen. *A:* A BNML colony grown from marrow and stained to visualize the presence of the lacZ gene by β-galactosidase activity (blue colour). *B & D*: Representative cytospin preparation of bone marrow cells from a rat injected with $1x10^7$ BNML cells (LT12NL15 subline) 21 days previously. *C & E:* Cytospin preparation of spleen cells from the same rat. *B & C:* Jenner-Giemsa stain. *D & E.* stain for β-galactosidase activity. Microscopic magnification A: 200x; B-E: 250x.


A
C

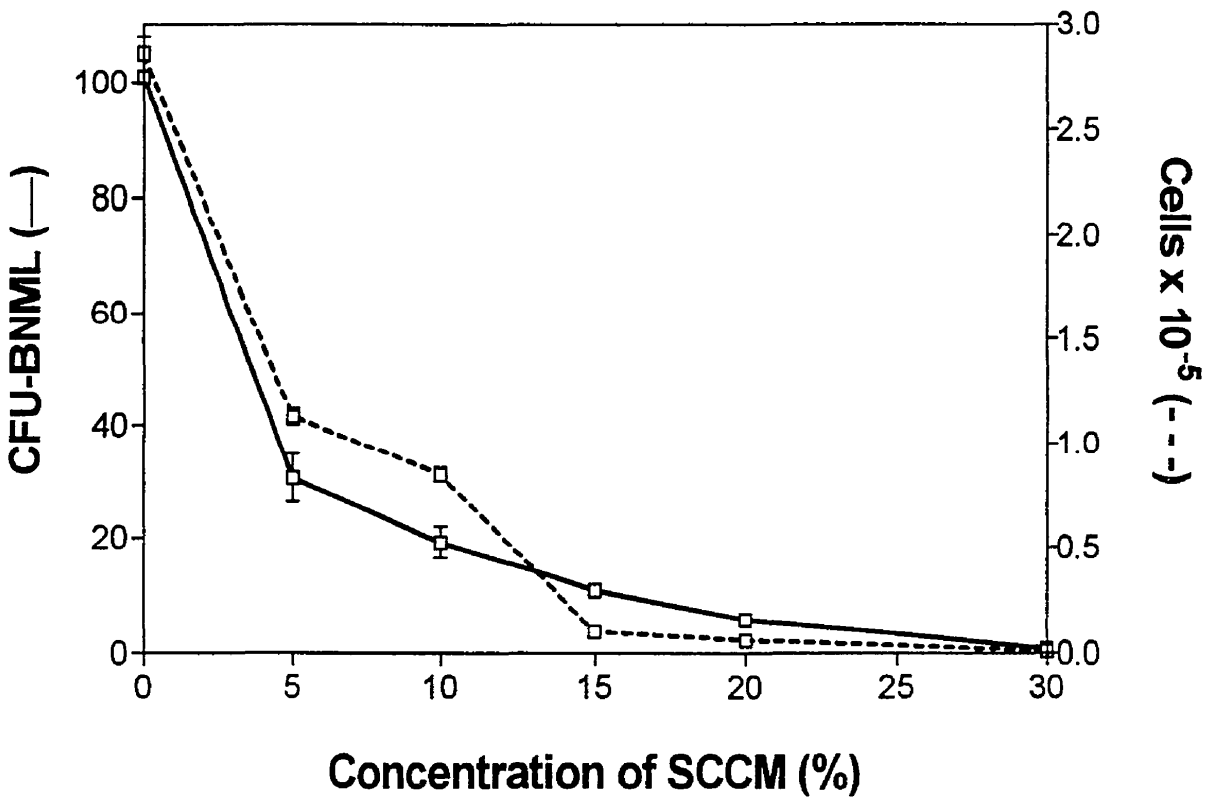
LEGEND TO FIGURE 3.

Comparison of leukemic and normal clonogenic cells in peripheral blood (PB), spleen (Spl), and bone marrow (BM) 18-21 days after iv injection of $1x10^7$ lacZ-containing BNML cells. Value bars are shown as the mean ($\pm$ SEM) of results obtained from 12-18 individual animals. Each data set was compared for statistical significance by the Kruskal-Wallis nonparametric ANOVA test. The differences were as follows: CFU-BNML: PB vs. Spl, $p<0.05$; PB vs. BM, $p<0.01$; Spl vs. BM, $p<0.001$. CFU-GM/M: PB vs. Spl, $p<0.001$; PB vs. BM, $p<0.05$; Spl vs. BM, $p<0.01$.


CFU-BNML/10^3 or CFU-C/10^5
100
80
60
40
20
0
CFU-BNML
CFU-GM/M
PB
Spl
BM
PB
Spl
BM
ORGAN

LEGEND TO FIGURE 4.

Dose-response relationship between concentration of SCCM and growth of BNML cells in liquid and semi-liquid cultures. Values shown are the mean (± SEM) of results obtained from 6-10 replicate experiments. Solid lines (——): CFU-BNML/$1.5x10^2$ cells plated in methyl cellulose; dashed lines (----): cell count resulting from $1x10^4$ cells/ml plated in liquid culture.


CFU-BNML (—)
Cells x 10-5 (- - -)
Concentration of SCCM (%)
0
20
40
60
80
100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
5
10
15
20
25
30

CHAPTER 3

EXPRESSION OF A THY-1-ASSOCIATED PROTEIN ON RAT MARROW STROMAL CELLS AND ITS POSSIBLE ROLE IN ADHESION OF MYELOID PRECURSORS

PREFACE

In Chapter 2 it was shown that in the intermediate stages of ***in vivo*** growth, BNML cells behaved differently in the bone marrow and the spleen. This raised the issue of whether the marrow stromal fibroblasts, and by implication the ST3 marker, contributed to this pattern of growth. Because adhesion of hemopoietic cells to stromal elements is a possible mechanism, I examined the possibility that ST3 might act as an adhesion molecule *in vitro*. In this chapter, we will demonstrate certain biochemical characteristics and actions of the ST3 antigen, which previous studies had shown to be borne on a cell surface molecule expressed on the predominant population of rat marrow fibroblastoid cells, but minimally on those from non-hemopoietic organs (e.g., lung).

ABSTRACT

The bone marrow microenvironment regulates the growth and differentiation of blood cells, and part of the process may involve a series of recognition steps between stromal cell subpopulations and hemopoietic precursors. Extending previous observations that an antibody called "ST3" reacts with most fibroblastoid cells derived from rat marrow, but with a minority of those growing from non-hemopoietic tissues, we tested whether the ST3 antigen might participate in adhesion reactions. Clonogenic myeloid precursors (CFU-G, -M, and -GM) bound to marrow fibroblasts, and the adhesion was blocked by ~ 40% after preincubation of the stromal cells with anti-ST3; erythroid precursors (CFU-E) bound minimally. Rat myeloid leukemia cells (BNML line) bound and were partially inhibited (~ 50%) by anti-ST3, but not by antibodies to CD11, CD18, ICAM-1, Thy-1(TIB103), or by an antibody ("ST4") reactive with fibroblasts from non-hemopoietic tissues. Late passage marrow fibroblasts, which did not express a detectable level of ST3 antigen, bound fewer BNML cells, and the binding was not inhibited by anti-ST3. Electrophoretic analysis and western blotting of marrow fibroblast-derived plasma membranes revealed that anti-ST3 reacted with a band coinciding with that stained by anti-Thy-1 (Ox-7); proteins immunoprecipitated by Ox-7 also bound anti-ST3. These results suggest that *in vitro* binding of normal and leukemic rat myeloid precursors to marrow stromal fibroblasts may be mediated in part by a region of the Thy-1 molecule recognized by the ST3 antibody.

KEY WORDS: Bone marrow, stromal cells, adhesion molecules, Thy-1 antigen, leukemia

INTRODUCTION

As hemopoietic progenitors self-renew, expand, and mature into functioning blood cells, they interact with the marrow microenvironment [1][2][3]. This process includes transmission of signals between cells, retention of precursors at sites of growth factor concentration, stabilization or augmentation of growth factor activity, metabolism of growth regulators, inhibition of growth or differentiation, and control over the exit of mature blood cells. Although evidence supports the notion of an anatomic "niche" where hemopoietic cells of different lineages and stages develop, the subpopulations of stromal cells (e.g., fibroblasts or sinusoidal endothelia) that may further specialize [4][5] to mediate such recognition steps have not been completely defined [6][7][8]. Thus, characterization of cell surface molecules that participate in these adhesion reactions will contribute to a better understanding of the structure-function relationships among hemopoietic and regulatory cells of the bone marrow microenvironment.

Previously, we described two murine monoclonal antibodies ("ST3" and "ST4") that distinguish between the predominant fibroblastoid cells that grow from rat bone marrow (ST3+/ST4-) and those that originate in certain non-hemopoietic organs (ST4+ cells > ST3+) [9][10]. The ST3 epitope is expressed also in the thymic cortex and on a subset of neurons [11]. Although anti-ST3 and anti-Thy-1 (Ox7) antibodies reacted with both common and distinct neuronal groups in immunohistologic preparations, the similarity in their staining patterns in other organs suggested that the two might be related. Because Thy-1 is a candidate cell adhesion molecule in the thymus [12][13], we investigated if the anti-ST3 antibody influenced the *in vitro* binding of normal and leukemic rat myeloid precursors to marrow stromal fibroblasts.

MATERIALS AND METHODS.

Bone marrow cells. Inbred male and female Brown Norway (BN) rats age 8-12 weeks were purchased from Charles River Canada (St. Constant, Québec), housed in polycarbonate plastic cages, and fed standard laboratory chow and water *ad libitum.* Femoral/tibial bone marrow was obtained by extrusion with a stream of medium expelled from a syringe through the centre of the bony shaft; nucleated cells were collected from the interface of a Ficoll-Hypaque gradient, and then washed three times with RPMI 1640 medium containing 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Stromal fibroblastoid cell cultures. Nucleated bone marrow cells ($2x10^6$/75 cm^2 flask) were cultured in 20 ml RPMI 1640 medium (supplemented with 10% FCS, 5% rat serum, 1% BSA, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) in a 37°C humidified incubator (5% CO2/95% air) for 2 wk without medium change, and then for another 2 wk with weekly half-medium changes. Homogeneous stromal monolayers were derived as previously described [10]. Briefly, adherent cells were detached by incubating with 0.05% trypsin-PBS (Gibco), recultured, and passaged at least four times. Hemopoietic cells were depleted by three cycles of treatment with mycophenolic acid (Sigma; #M5255) at a final concentration of 5 μg/ml; macrophages were depleted by antibody-dependent complement-mediated cytotoxicity.(BN35 antibody; murine monoclonal to rat myeloid cells, including CFU-G/M). After 4-8 passages, fibroblastoid cells were removed from the flasks by a 5-8 min incubation in trypsin-PBS at 37°C. After two washes with regular medium, they were distributed into either 96-well culture dishes at $5x10^3$ cells/well, 4-well chamber slides (Lab-Tek; #177437) at $1x10^4$ cells/well, or to 6-well culture dishes at $1x10^5$ cells/well, and cultured for 3-5 days prior to immunologic analysis or assay of cell adhesion after the growth had become 50-80% confluent by visual inspection. Pilot experiments showed that these cells strongly expressed ST3, Thy-1/TIB103, and ROS 107, weakly VLA-4, but not LFA-1(α or β chain), ICAM-1, MHC class II, ST4, or D46 antigens (antibodies summarized in Table 1). Derivation and characterization of fibroblastoid cell lines from rat bone marrow and lung have been described elsewhere [14].

Enzyme linked immunosorbant assay (ELISA) and immunohistochemical staining. Cells prepared as described above were fixed in 0.05% glutaraldehyde in PBS (pH 7.4) for 10 min. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 for 30 minutes. After rinsing 3x with PBS, the cells were incubated for 20 minutes in 0.1% gelatin/PBS, and again rinsed 3x with PBS prior to a 30 min incubation with the primary murine antibody at the appropriate dilution. After rinsing with 0.1% BSA/PBS, another 15 min incubation with 0.1% gelatin/PBS, and another 3 rinses with PBS, the preparation was incubated for 30 min with peroxidase-conjugated goat anti-mouse antiserum (anti-IgG + IgM heavy and light chains; Kirkegaard Perry [#041809], Rockwood, Ontario; diluted 1:100 for chamber slide staining or 1:500 for ELISA in 0.1% BSA/PBS). After rinsing, the substrate reaction was as follows: (1) Chamber slide histology: AEC (0.2 mg/ml 3-amino-9-ethylcarbazole, Aldrich Chemical Co.) in the AEC buffer (0.10 M acetate; pH 5.1) was added to each slide for 10-15 minutes at room temperature, and then rinsed with PBS, counterstained with Mayer's hematoxylin for 30 min, rinsed with warm tap water, and mounted in glycerol/PBS. (2) ELISA assay: Preparations were incubated with the peroxidase substrate (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); Sigma Chemical Co.; A-4798 #112H8903) until the absorbance of the ROS 107 control was 0.300 (30-45 minutes), and the absorbance was measured with a microplate ELISA reader. Negative control antibodies included subclass specific murine monoclonals of irrelevant specificity; positive controls included uncharacterized murine antibodies selected for broad reactivity with rat tissues.

Adhesion assays. The stromal cell monolayer was incubated at 37°C for 50 min with the stated antibody at a dilution that had been shown to result in optimal staining by indirect immunoperoxidase. After two washes in warm 1640 medium, $1x10^6$ bone marrow or BNML cells (depending on the experiment) were added over the stromal cell monolayer (or for controls, the culture dish without stromal cells), and further incubated for 3 h at 37°C. For some experiments, the hemopoietic cells had been dye-labelled (see below). In assays designed to measure binding of hemopoietic precursors, cells were added over the stromal cell monolayer in 1 ml RPMI 1640 medium containing 5% FCS; $1.5x10^5$ cells were added for assay of CFU-G/M or CFU-E and $3x10^2$ for assay of CFU-

BNML. After gentle agitation for one min the non-adherent cells were collected, the stromal monolayers were washed twice with warm medium, the decanted liquid pooled with the original supernatant, and the free cells either enumerated by flow cytometry or assayed for clonogenic precursors as previously described [10].

Clonogenic cell assays. Clonogenic assays (CFU-G/M, CFU-E) were performed with non-adherent cells by standard methods, as previously described [10]. Briefly, 1 ml of the culture system consisted of Iscove's Dulbecco's medium (IMDM; GIBCO, Grand Island, NY), supplemented with either 30% FCS/1% BSA/5% spleen cell-conditioned (SCCM for CFU-G/M) or 1 u/ml of erythropoietin (for CFU-E; Step III, purified from sheep, Connaught Laboratories, Willowdale, Ontario, No. 1501-5-7), $5x10^{-5}$ M 2-mercaptoethanol and 0.8% methyl cellulose (4000 centipoise, Fisher Cat.#M-352). The 35-mm dishes were cultured at 37°C in an atmosphere containing 5% CO_2. Clonogenic assay of stroma-adherent hemopoietic cells was performed by overlaying the washed culture dishes with 2 ml of culture medium containing the above ingredients. The CFU-BNML assay system was similar to the normal CFU assay, except that neither SCCM, nor erythropoietin was added. The CFU-E were counted on day 2, and CFU-G/M and CFU-BNML on day 7, using an inverted microscope; a colony was defined to be >50 cells.

The SCCM was prepared by culturing nucleated spleen cells ($1x10^6$/ml) in RPMI 1640 medium containing 10% FCS, 1% BSA, 2 mM L-glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml phytohemagglutinin (PHA-L Pure; E.Y. Labs, San Mateo, CA). After 4-5 days of incubation at 37°C in 5% CO_2/95%, the supernatant was centrifuged at 1200 rpm for 30 minutes and stored at -20°C. The concentration of SCCM employed (5%) was that previously shown to cause maximal stimulation of colony formation of CFU-G/M.

Labelling of indicator cells. Normal nucleated bone marrow cells or rat myeloid leukemia cells (BNML line, clone LT12n115 containing the lacZ gene, gift of Dr. A. Hagenbeek and P. Hendrikx, Erasmus University, Rotterdam, The Netherlands) were labelled with the fluorescent dye "CellTracker Green" according to instructions provided by the supplier (Molecular Probes, Inc, C-2925). Briefly, 10 μM (final concentration)

CMFDA (5-chloromethylfluorescein diacetate) was added to $5x10^7$ cells in a total volume of 1 ml 1640 medium, and incubated at 37°C for 20-30 minutes. After centrifugation, fresh dye-free medium was added and cells were incubated for another 30 minutes, washed twice, and resuspended for further use. Flow cytometric analysis was performed using a FACScan (Epics Profile).

Preparation and electrophoretic analysis of plasma membranes: Bone marrow fibroblastoid cells were expanded into T150 flasks or 150 mm^2 petrie dishes; $1x10^9$ were harvested by scraping, resuspended in 50 ml PBS, and centrifuged at 100xg for 6 min. After two further washes, they were resuspended in 40 ml PBS/5 mM $MgCl_2$ (pH 7.4) containing the following protease inhibitors: 6.25 μg/ml aprotinin, 4 mM iodoactetamide, 2 mM PMSF, 2.5 mM EDTA, 1 mM N-ethyl-maleimide, 1 mM benzamidine and 0.1 mM 6-aminocaproic acid, and disrupted by nitrogen cavitation at 450 psi. This suspension was then centrifuged at 200xg for 10 min; the supernatant was centrifuged at 12,400xg (ss34 Sorvall rotor) for 15 min, and that supernatant was centrifuged at $1.25x10^5$xg (Beckman 50Ti rotor) for 1 h. The pellet containing the plasma membranes was suspended in 5 ml PBS containing 1% Triton X-100 and the protease inhibitors, and resuspended using a Dounce homogenizer (20-25 strokes). After further centrifugation at 13,000xg (Eppendorf minifuge) for 10 min, the supernatant was retained for western blot analysis immediately or after storage at -20 °C.

Immunoprecipitation: Protein-A linked to Sepharose CL-4B beads (0.1 g; Pharmacia, #17-0780-01) were hydrated in 1 ml of 100 mM potassium phosphate buffer, pH 7.0 for 1 h, centrifuged and resuspended in 100μl of potassium phosphate buffer. Ox-7 monoclonal antibody (25 μl) was added to the bead suspension and incubated at 4°C for 16 h. After a single wash with 0.5% Triton X-100 (0.15 M NaCl, 50 mM Tris-HCl, pH 7.5) and twice more with 0.05% Triton X-100 (0.15 M NaCl, 50 mM Tris-HCl, pH 7.5), a 250 μl aliquot of the membrane preparation as incubated with the Ox-7-bound beads at room temperature for 1 h. The mixture was centrifuged as above, and the supernatant retained for western blot analysis. The beads then were washed 3x with 0.05% Triton buffer, after which 200 μl of 2x PAGE loading buffer (4% SDS 20% glycerol, 0.002% bromophenol blue and 120 mM Tris-HCl, pH 6.8) was added and the

suspension heated to 85°C for 10 min. The suspension was centrifuged, and aliquots of the supernatant were subjected to SDS-polyacrylamide gel electrophoresis according to the technique of Laemmli et al using a Biorad Mini apparatus [15]. The proteins then were transferred to a nitrocellulose membrane as described by Towbin et al [16] using a Biorad Mini Protean Transblot apparatus (100 volt for 60 min); the transfer buffer was modified to contain 8.3 mM Tris/64 mM glycine/pH 8.3 and 20% methanol.

After transfer, the nitrocellulose membrane was washed three times in PBS, and then incubated overnight at 4°C in blocking buffer containing Tris-buffered saline (NaCl 138 mM; KCl 3 mM; Tris 25 mM; pH 8.0, 5% skim milk, and 0.05% Tween 20). The membranes then were incubated for 2 h with a predetermined dilution of the monoclonal antibody (ascitic fluid), washed with the blocking buffer, and then incubated with the second antibody (1/5000, rabbit anti-mouse IgG; Jackson/Biocan) for 90 min. After further washing with blocking buffer, the membranes were incubated with the third antibody (1/3000, goat anti-rabbit IgG conjugated to horseradish peroxidase; Biorad) in blocking buffer for 1 h. After the final wash, the color was developed using the NBT/BCIP substrate kit (Biorad).

Statistical analysis. Unless otherwise stated in the text, the data in Figs 2-6 were analyzed by one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons test using a personal computer and INSTAT software (GraphPad Software, San Diego CA).

RESULTS.

Relation of the ST3 antigen to rat Thy-1. Gel electrophoresis of marrow stromal cell membranes and subsequent immunoblotting showed that the mobility of the major band stained by anti-ST3 (27.2 - 28.6 kDa) coincided with that stained by anti-Thy-1 (Ox-7) (Fig 1A). To indicate if the two antibodies might bind to epitopes on the same protein, detergent extracts of stromal cell membranes were subjected to immunoprecipitation with Ox-7, and then electrophoresed, transferred to a nitrocellulose membrane, and reacted with the antibodies. This demonstrated that both anti-ST3 and anti-Thy-1 (TIB103) either bound to an epitope carried by the Thy-1 protein, or to other proteins that co-immunoprecipitated with Ox-7 (Fig. 1B).

Effect of the ST3 antibody on adhesion of normal bone marrow and leukemic cells to stromal fibroblasts. Because most bone marrow fibroblasts express the ST3 antigen, we investigated whether it might participate in adhesion reactions with hemopoietic cells. In culture dishes treated with only BSA (i.e., lacking a stromal layer), 79 $\pm$ 1.6% (SEM) of nucleated bone marrow cells remained in suspension after incubation; presumably macrophages and other myeloid cells (21%) adhered to the plastic. In dishes containing stromal cells, however, 41-46% were recovered in the non-adherent fraction, i.e., $\sim$ 35% more adhered when stromal cells were present. As shown in Fig 2, none of the antibodies tested (anti-CD11/18, ICAM, Thy-1/TIB103, ST4, or.ST3) altered this adhesion. In contrast, leukemic cells (BNML line) did not adhere significantly to the culture dish unless stromal cells were present, in which case 47.1 $\pm$ 2.4% (vs. 93% for BSA control) remained in suspension. This pattern was not altered by any of the antibodies tested except for anti-ST3, which resulted in 77.9 $\pm$ 2.3% of cells remaining in the non-adherent fraction, indicating that the anti-ST3 antibody inhibited the stroma-dependent adhesion of BNML cells to the stromal layer by approximately 67%.

Effect of the ST3 antibody on the adhesion of clonogenic precursors. The observation that anti-ST3 inhibited the adsorption of BNML cells (but not of unfractionated normal marrow cells) to the stromal layer suggested that the binding might be selective for a subpopulation of hemopoietic precursors. As shown in Figure 3, 6.5 $\pm$ 0.7% of clonogenic BNML cells adhered to the culture dish in the absence of a

stromal layer ("BSA"). In culture dishes containing stromal cells that had not been pre-incubated with any antibody, 43 ± 0.5% of the clonogenic cells adhered. Again, this adhesion was not altered by preincubation with any antibody other than anti-ST3, which decreased the binding to 26 ± 0.7%, leaving 72 ± 1.0% in suspension. This indicates that the anti-ST3 antibody inhibited the adhesion of clonogenic BNML cells to the stromal layer by approximately 40%. If BNML cells (instead of the stromal layer) were preincubated with anti-ST3, the adsorption was not decreased (Table 2), although it was partially blocked by anti-VLA-4.

Similar experiments were performed on the clonogenic cells of bone marrow derived from normal rats. In the presence of a stromal cell layer, 28 ± 0.6% of the myeloid colonies (CFU-C) adhered (Fig. 4), and preincubation of the stromal cells with the anti-ST3 antibody decreased this to 14 ± 1.0% (i.e., by ~ 50%). Concurrently, preincubation with anti-ST3 increased the CFU-C in the nonadherent fraction from 52 ± 2% to 86 ± 1.4%. However, neither the control D46 nor ST4 antibodies had any affect. Under similar conditions, fewer CFU-E were removed by the stromal cells (81 ± 2% in the non-adherent fraction), and the pattern was not altered by anti-ST3.

Adhesive properties of stromal cells in relation to their expression of ST3 antigen. Previous experiments had shown that after repeated passage the ST3 antigen was no longer detected on marrow fibroblastoid cell lines by immunohistologic methods. Comparison of the relative expression of Ox-7/Thy-1 and ST3 antigens on primary and late- passage marrow fibroblasts is shown in Figure 5. Although expression of Ox-7 decreased to approximately one half the level detected on primary marrow fibroblasts, it remained within the limits of detection by both the ELISA assay and immunohistologic staining (not shown). In contrast, the ST3 antigen was not detected above background level in the late passage cells. This provided a means to further evaluate the possible contribution of the ST3 epitope to the adhesion reaction. As shown in Figure 6, fewer BNML cells adsorbed to the late-passage stromal line (81 ± 3% remained in the suspension) than to primary marrow fibroblasts (Fig 3), and none of the antibodies tested, including anti-ST3, decreased the binding.

Other cells that express the ST3 antigen would be expected to bind myeloid

precursors. We have shown that ST3 is expressed also by the fibroblastoid cells that form within subdermal hemopoietic ossicles induced by implantation of bone morphogenetic protein (An *et al,* 1996). The results of Figure 7 demonstrate that such stromal cells bind CFU-C as well as do fibroblasts grown from authentic femoral marrow.

DISCUSSION.

In these experiments, the binding of both normal and leukemic myeloid precursors to primary marrow fibroblasts was partially inhibited by the anti-ST3 antibody, but not by a control IgM (D46) or by an anti-Thy-1 IgM (TIB103). Furthermore, a late-passage stromal cell line that no longer expressed detectable ST3 antigen bound fewer leukemic cells (BNML line), and the adhesion was not decreased by preincubation with anti-ST3. Analysis of plasma membrane extracts of primary marrow fibroblasts showed that the polypeptide carrying the ST3 epitope had an electrophoretic mobility identical to that carrying Thy-1 (Ox-7 or TIB103), and was co-precipitated with an IgG antibody to Thy-1 (Ox-7). Preliminary experiments suggest that the ST3 epitope may be sensitive to N-glycanase, implying that it might be part of an oligosaccharide moiety, but proof of this will require direct demonstration of binding to the purified glycopeptide. Together, these results raise the possibility that a receptor on the surface of rat myeloid.precursors could recognize a site on the Thy-1 glycoprotein, and either bind to it directly or activate other mechanisms to effect binding. Experiments transfecting the Thy-1 gene into cell lines with different glycosylation patterns could confirm or refute this hypothesis.

The observation that ST3 does not completely inhibit the binding of normal and leukemic clonogenic cells is consistent with the findings of other investigators. Evidence exists that multiple cell surface molecules participate in adhesion reactions between hemopoietic precursors and stromal cells which, in addition to maintaining topographic relationships, can generate further signals in an "adhesion cascade" [17]. Examples of this include an increase in the basal level of VCAM-1 on stromal cells in long-term marrow culture after addition of IL-1β, TNFα, or IL-4, and a decrease after addition of TGF-β [4][18]. Adhesive function also can be modulated, as shown by the increased

avidity of binding to $\alpha_4\beta_1/\alpha_5\beta_1$ integrins following exposure of hemopoietic lines to stem cell factor [19]. Effective binding may involve a sequence of reactions, as observed with cell lines that bind by an initial α_4 integrin-dependent phase followed by α_4-independent steps [20]. Signals generated subsequent to adhesion include tyrosine phosphorylation and proliferation in B lymphocytes and myeloma cells after engagement of VLA-4 by fibronectin [21][22]; F-actin polymerization and homotypic cell aggregation through secondary activation of β_2 integrins after binding of anti-CD34 to its ligand [23]; and fibronectin/VLA-5 binding through a *c-kit* kinase-dependent step in mast cells at a concentration of stem cell factor 100-fold lower than required to induce replication [24].

These reactions may lead to physiologically relevant mechanisms. By the controlled expression of constellations of adhesion molecules, marrow microenvironments favorable to the expansion of a given lineage or stage of maturation may develop. Illustrating this is the binding of CFU-E and pre-B cells to fibronectin, which decreases as the cells mature and egress from the marrow [25][26][27]. Committed precursors (BFU-E and CFU-GM) attach directly to the heparin-binding domain of fibronectin through a mechanism inhibited by prior treatment with chondroitinase A/B/C, β-D-xyloside, or antibodies to α_4 or β_1 integrin chains [28]; BFU-E and lymphoblastoid lines also can use the VLA-4/VCAM-1 pair [29][30]. Hemonectin, present in bony tissue of fetal mice prior to the onset of granulopoiesis, is found in hemopoietic organs of the adult and preferentially binds CFU-GM [31][32]. Multipotent progenitors also use a variety of cell adhesion molecules, as demonstrated by the stromal/$CD34^+$ cell binding through the $\alpha_4\beta_1$- $\alpha_5\beta_1$ /VCAM-1 system [33][34][35][36], in addition to mechanisms that are not blocked by free RGD peptides or antibodies to fibronectin and laminin but are inhibited by treatment with heparinase, chondroitinase, and hyaluronidase [37]. Components of the extracellular matrix, such as specialized forms of heparan [38] and chondroitin-4-sulfates [39][40], may generate local gradients of growth factor concentration [41][42], and may act with other ligands, as observed when T lymphocytes adhere to endothelial cells through a multivalent complex of VCAM-1/MIP-1β bound to heparin or the CD44 hyaluronan receptor.

Although the function of Thy-1 remains uncertain, it appears to participate in

relevant signalling events in several tissues. Its distribution includes primitive hemopoietic progenitors (CFU-S_{12}), as well as committed macrophage and granulocyte precursors (CFU-G/M) [43]. In both mouse and man, it is expressed on early stage stem cells, raising the speculation that it may deliver an anti-proliferative signal to maintain the resting state [44][45]. As cells of the myeloid lineage mature, Thy-1 is lost, and in the lymphoid system the inverse correlation between its expression and maturity is observed on both B and T cell subsets [46]. Being a phosphoinositol-linked glycoprotein of 24-25 kDa, of which approximately a third consists of N-linked oligosaccharide [47], its composition can vary in different tissues of the same species or be altered at different stages of differentiation, as noted when the molecule becomes more heavily sialylated as thymocytes mature [48]. The three glycosylation sites have been compared in neural Thy-1 from rat, human, and mouse. Although the amino acid sequences were not identical, the overall structures of the N-glycans were similar, suggesting that the carbohydrate might contribute a functional component to the polypeptide carrier [49]. Indeed, Williams has speculated that Thy-1 may be an adhesion molecule, based on its being a single domain of the immunoglobulin superfamily, which includes molecules of the immune response (e.g., CD3, MHC class I and II) and other systems where adhesion reactions contribute to function (e.g., N-CAM, CEA, PDGF receptor) [50].

In the immune system, Thy-1^+/$CD3^+$ thymocytes proliferate after engagement by anti-Thy-1, and may be activated through a signal pathway different from that initiated by anti-CD3ϵ [51]. In human lymphoid cell lines, cross-linking of Thy-1 causes immediate phosphatidylinositol turnover and influx of extracellular Ca^{++} [52]. In murine thymocytes, signalling with the two classes of antibodies resulted in tyrosine phosphorylation, and induction of apoptosis, mimicking the signal for negative selection [53]. Immunoprecipitation of Thy-1 from detergent extracts of chemically cross-linked T cells coprecipitated CD45 tyrosine phosphatase and MCH class I, suggesting that the T cell activation may occur through inhibition of this activity [54]. Direct evidence that Thy-1 might participate in cellular adhesion reactions derives from the observation that binding of murine T lymphoma cells to a thymic epithelial line was partially inhibited by soluble Thy-1 molecules and by Fab' fragments of anti-Thy-1 antibodies [12][13].

Further studies showed that this reaction could be blocked by heparin, which also can bind Thy-1, but not by chondroitin, keratan, or heparan sulfates [55]. In adult neurons, Thy-1 proteins can form multimers which may inhibit neurite extension to astrocytes, or stabilize neuronal connections *in vivo* [56][57]. In the lung, a subset of fibroblasts expressing Thy-1 and receptors for IL-4 shows a characteristic pattern of collagen secretion in response to IL-1 and TNF [58].

The results presented here do not enable us to speculate on what might be the *in vivo* significance of the observed *in vitro* binding, or what might be the co-receptor for the ST3 epitope on hemopoietic cells. However, the expression of ST3 on >95% of fibroblasts grown from the marrow, but on only a minority of those grown from other organs [9], argues that the antigen might play a role in marrow function; the reactivity of anti-ST3 with a subpopulation of Thy-1 molecules raises the possibility that it might do so in the context of a Thy-1-mediated signal. The observation that the ST3 epitope decreased out of proportion to the loss of Ox-7 reactivity with prolonged passage in culture raises the possibility that the antigen might be regulated by other stimuli (e.g., local factors and/or cytokines), as reported for expression of matrix components in long-term marrow cultures [59][60] or for CD34 on microvascular endothelial cells [61]. Further studies to modify the oligosaccharide and peptide regions of the Thy-1 molecule and then observe subsequent changes in ST3 expression and cell adhesion should clarify these issues.

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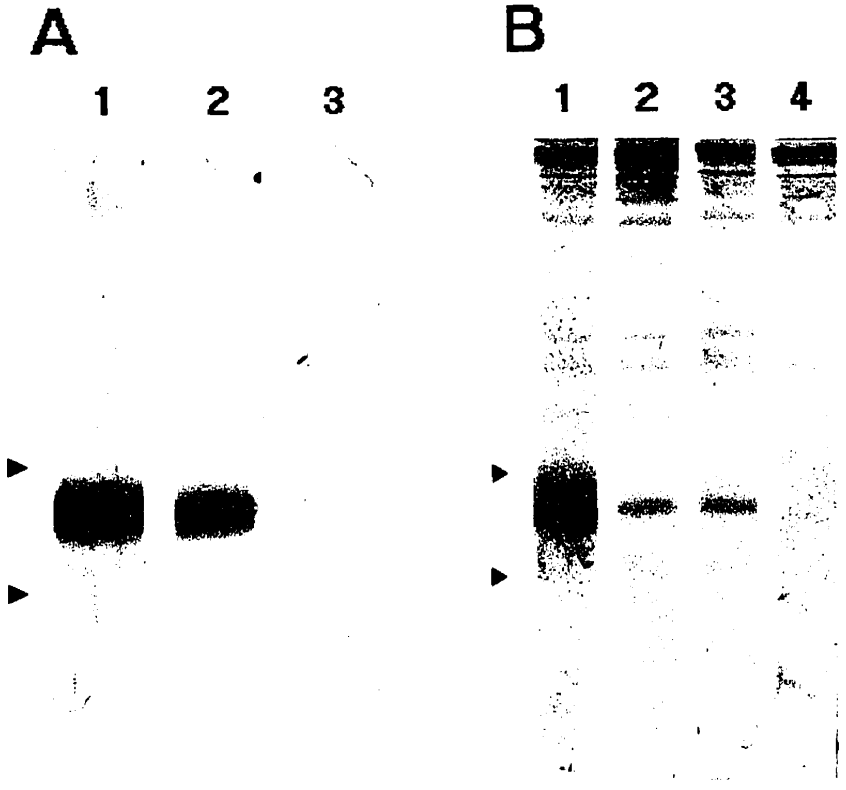
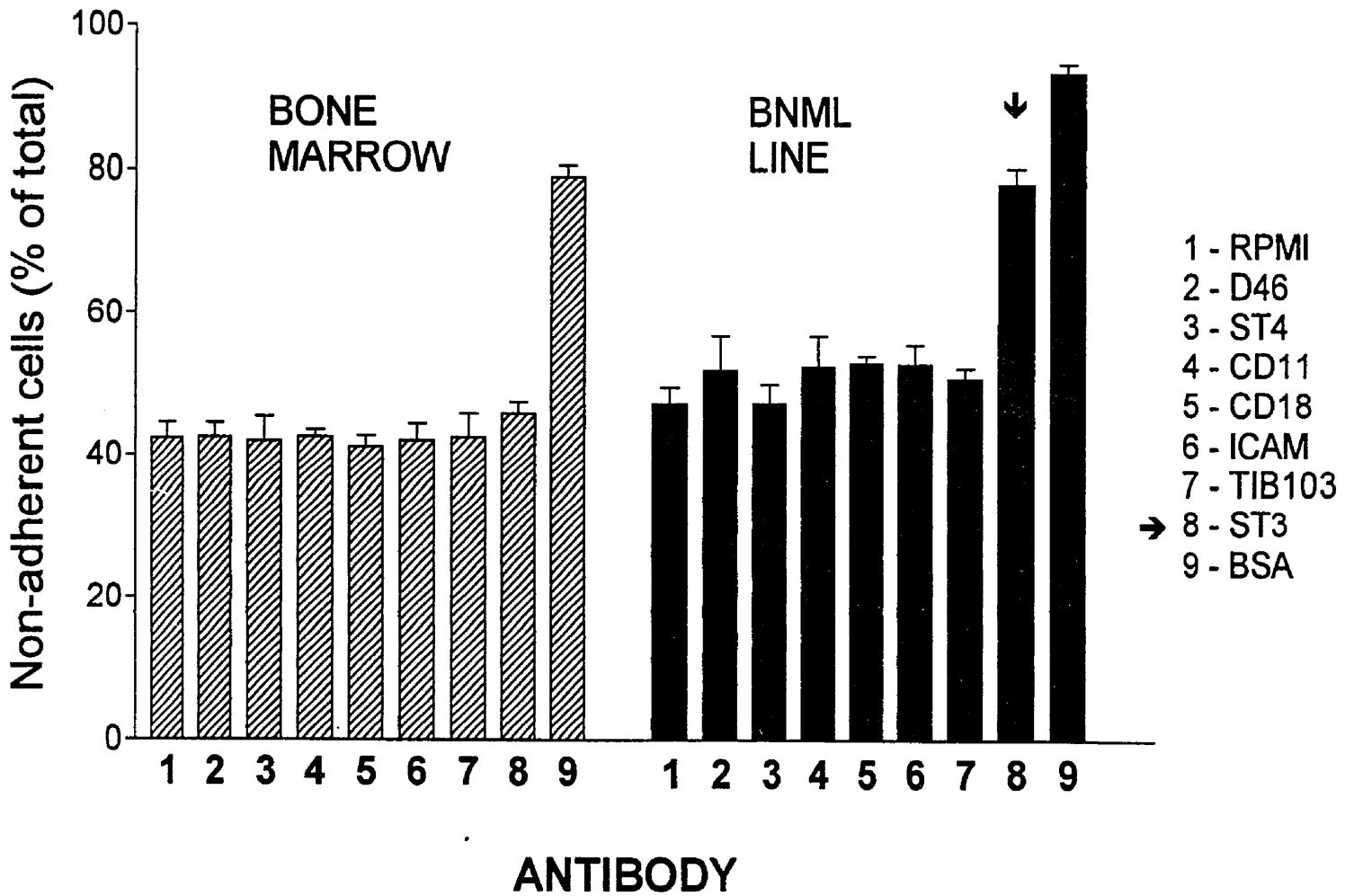
LEGEND TO FIGURE 1.

Immuno/western blotting of proteins from polyacrylamide gel electrophoresis of detergent extracts of rat marrow stromal fibroblasts. (A) Unmanipulated detergent extracts applied to electrophoresis under nonreducing conditions. lane 1: anti-Thy-1 (Ox-7); lane 2: anti-ST3; lane 3: D46 (negative control IgM) (B) Detergent extract subjected to immunoprecipitation with Ox-7, electrophoresed, and then blotted with the noted antibodies. lane 1: Ox-7; lane 2: anti-ST3; lane 3: TIB103; lane 4: D46. Arrow heads denote the mobilities of the 31.0 and 21.5 kDa marker proteins.


A
1
2
3
B
1
2
3
4

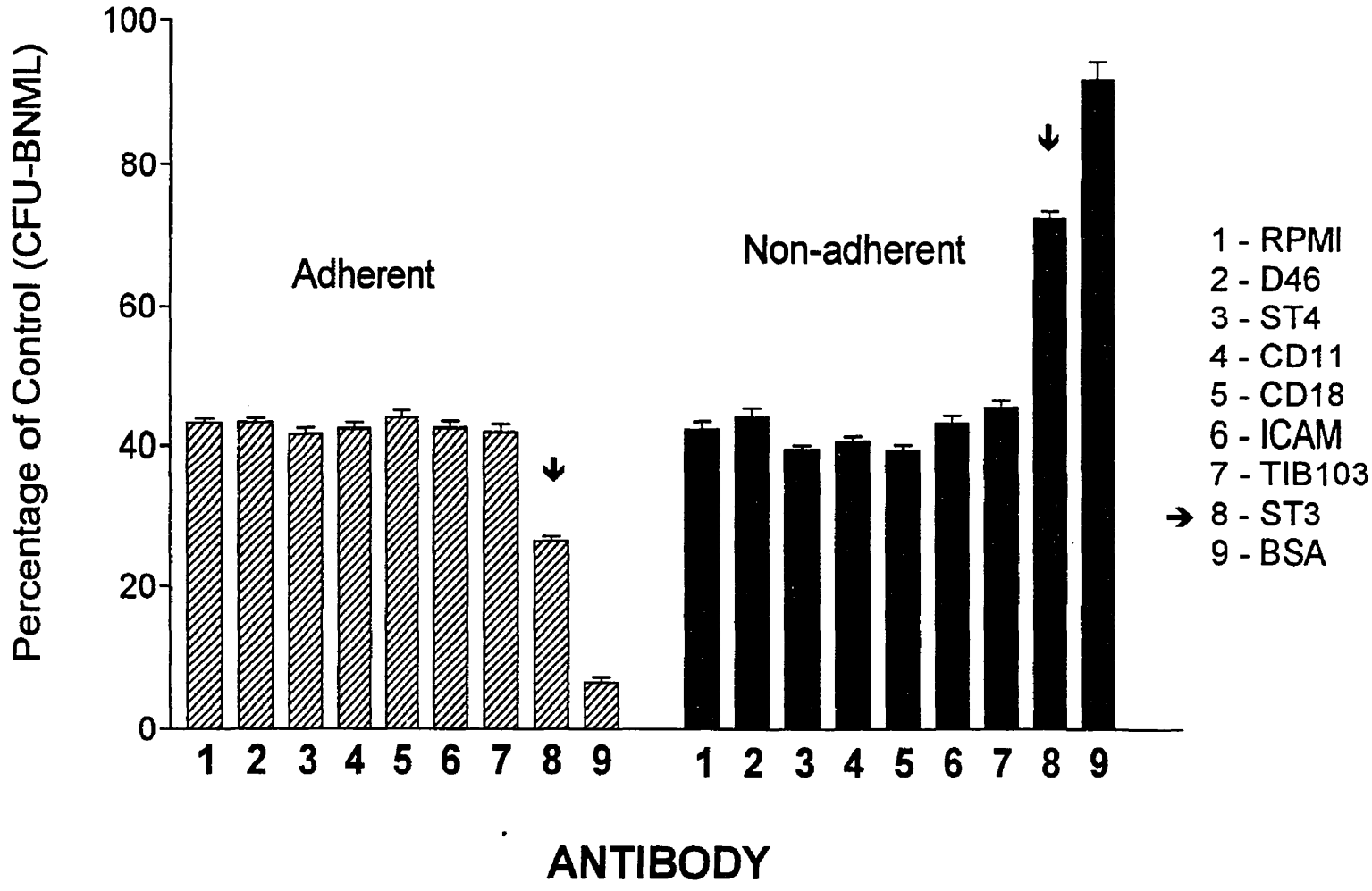
LEGEND TO FIGURE 2.

Effect of pre-incubation of the stromal cell layer with various antibodies (see Table 1) on subsequent adsorption of hemopoietic cells. Unfractionated nucleated bone marrow cells (hatched bars) or BNML myeloid leukemic cells (solid bars) were labelled with CMFDA dye, and incubated with a monolayer of primary fibroblastoid cells derived from rat marrow. The values denoted as "BSA" represent control dishes that did not contain stromal cells, but were incubated only with BSA prior to addition of hemopoietic cells. The non-adherent cells were recovered and enumerated by flow cytometry. Each assay was performed in triplicate with 3-5 independent preparations of stromal cells, each analyzed on different days. The results are expressed as the mean $\pm$ S.E.M., normalized as to % of the number of cells initially added. In experiments with *bone marrow cells* applied to the stromal layer, none of the antibodies resulted in any significant change from the RPMI control ($P > 0.05$). In experiments with *BNML cells*, only anti-ST3 increased the number of cells in the non-adherent fraction (ST3 vs. RPMI, $P < 0.001$ by ANOVA test).


Non-adherent cells (% of total)
100
80
60
40
20
0
BONE MARROW
BNML LINE
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
ANTIBODY
1 - RPMI
2 - D46
3 - ST4
4 - CD11
5 - CD18
6 - ICAM
7 - TIB103
8 - ST3
9 - BSA

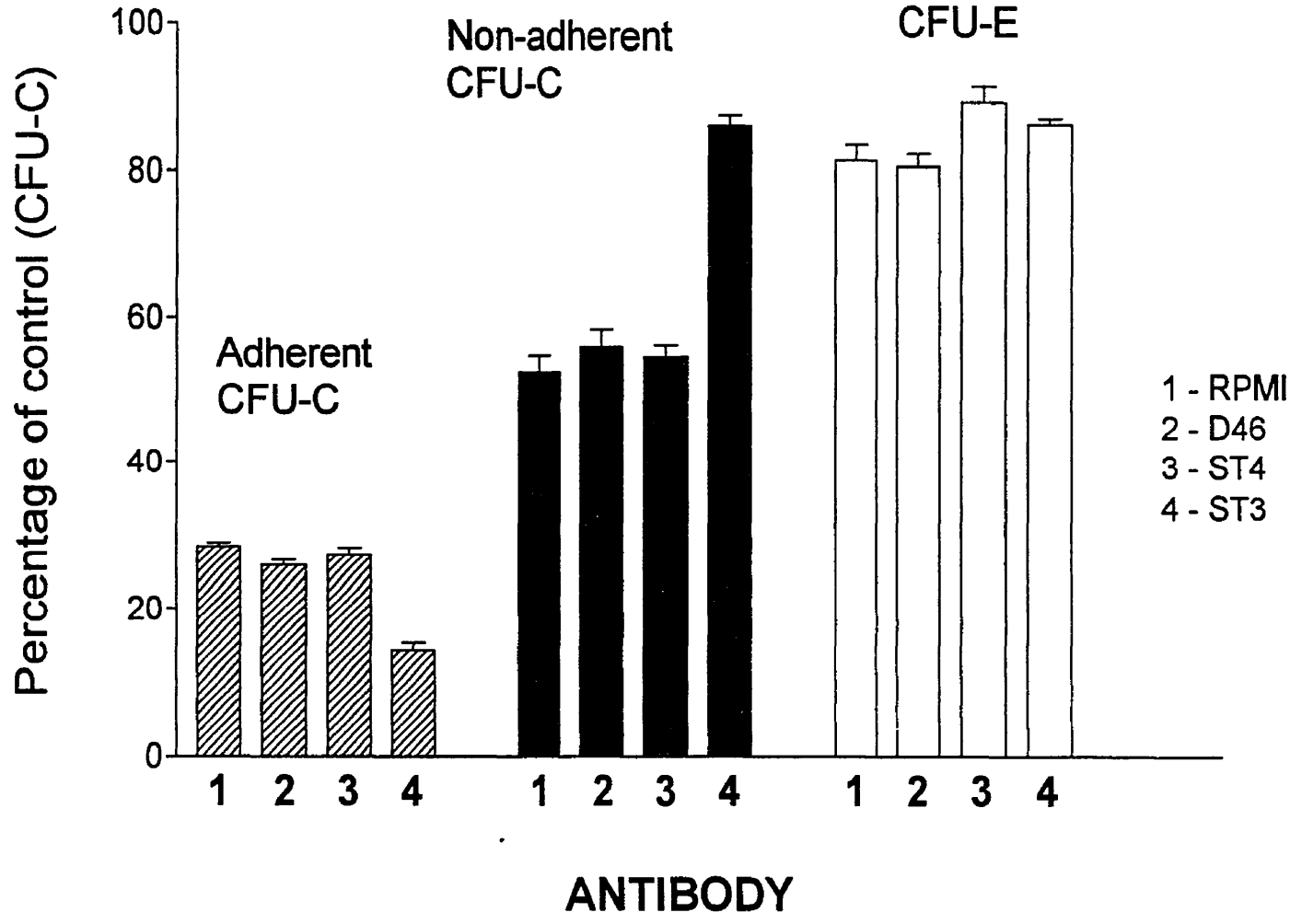
LEGEND TO FIGURE 3.

Effect of pre-incubation of the stromal cell layer with various antibodies on subsequent adsorption of clonogenic BNML myeloid leukemic cells. The cells that adhered (hatched bars) and those that remained in suspension (solid bars) were measured as described in the "Methods". The clonogenic efficiency of BNML cells used in these experiments was 75 $\pm$ 9% (SEM). The results in the figure are expressed as percent of control, 100% being the frequency of clonogenic cells in the initial marrow sample that had not been incubated with either the stromal cells or the cell-free culture dish. The bars labelled "BSA" refer to binding to the BSA-coated culture dish in the absence of a stromal layer. "RPMI" refers to the media control in the absence of added antibody, but with a stromal layer present in the culture dish; the other bars refer to values obtained when the stromal cells had been preincubated with the noted antibody. Each assay was performed in duplicate or triplicate with at least 3-5 independent preparations of stromal cells analyzed on different days. In comparison to all other antibodies, the effect observed for anti-ST3 on the inhibition of adhesion was significant at the $P < 0.001$ level for experiments directly measuring both the adherent and non-adherent clonogenic cells.


Percentage of Control (CFU-BNML)
100
80
60
40
20
0
Adherent
Non-adherent
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
ANTIBODY
1 - RPMI
2 - D46
3 - ST4
4 - CD11
5 - CD18
6 - ICAM
7 - TIB103
8 - ST3
9 - BSA

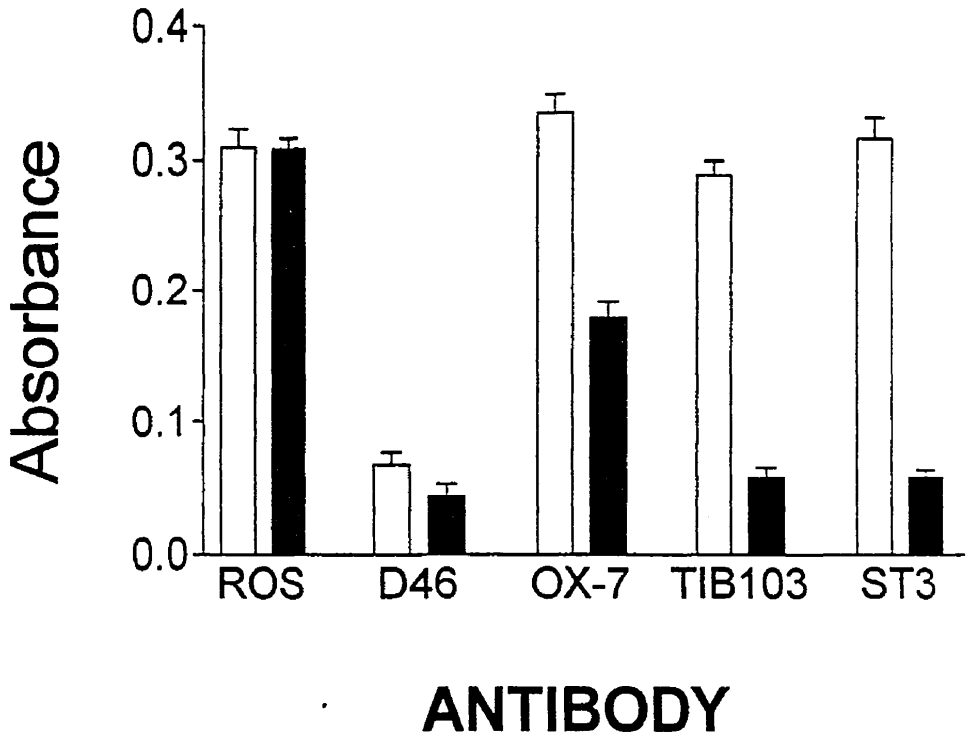
LEGEND TO FIGURE 4.

Effect of pre-incubation of the stromal cell layer with various antibodies on subsequent binding of normal clonogenic bone marrow cells. Cells (1.5×10^5) were incubated with a monolayer of primary fibroblastoid cells derived from rat marrow, and the CFU-C (CFU-G+M+G/M) that adhered (hatched bars), that remained non-adherent (solid bars), and the non-adherent CFU-E (open bars) were measured. "RPMI" refers to media control without antibody; the other values denote the antibody with which the stromal cells had been incubated. Each assay was performed in duplicate with at least 3-5 independent preparations of stromal cells analyzed on different days. Data are expressed as the mean $\pm$ SEM, normalized to percent of control; control was the number of colonies recovered after incubation of cells in culture dishes lacking a stromal layer. In the CFU-C assay, the differences between D46 or ST4 and the RPMI control were not significant ($P > 0.05$), but the differences between ST3 and the others were significant ($P < 0.001$). In the CFU-E assay, ST3 did not differ significantly from the others ($P > 0.05$).


Percentage of control (CFU-C)
100
80
60
40
20
0
Adherent CFU-C
Non-adherent CFU-C
CFU-E
1 - RPMI
2 - D46
3 - ST4
4 - ST3
1 2 3 4
1 2 3 4
1 2 3 4
ANTIBODY

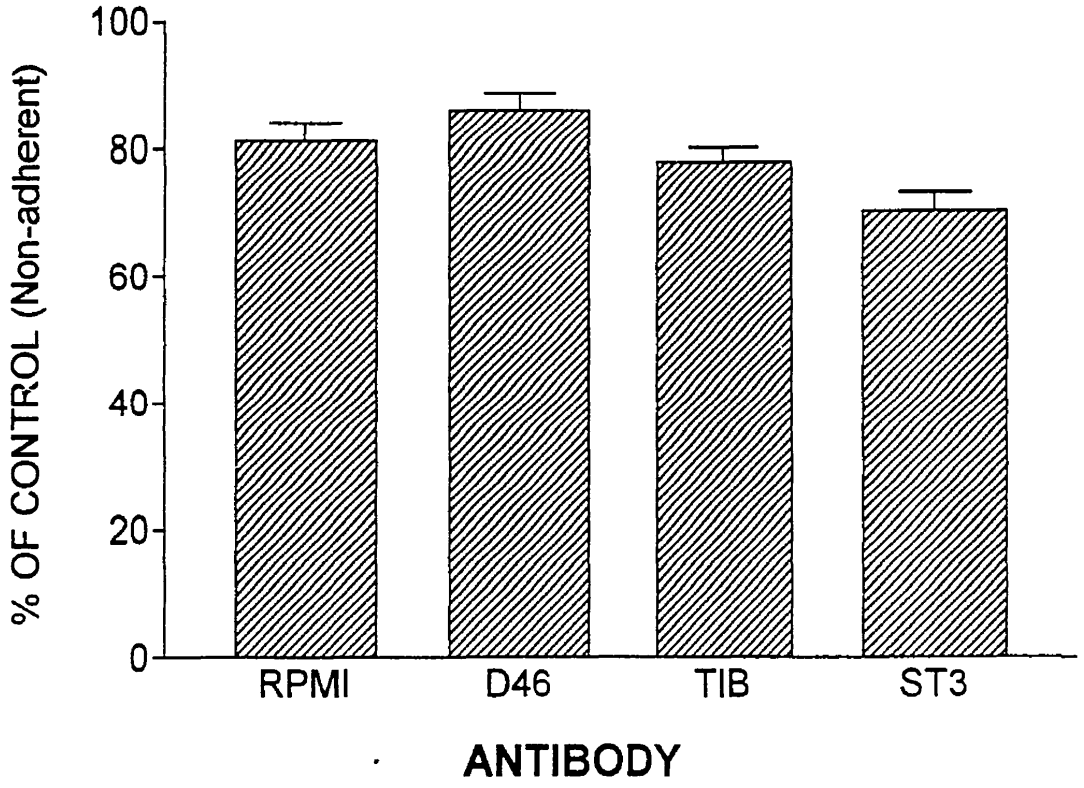
LEGEND TO FIGURE 5.

Comparison by ELISA assay of antigen expression on primary (open bars) and multiply-passaged (solid bars) stromal fibroblastoid cells. The "late" passage line was used after being further subcultured another 21-25 times. In each assay, the optical absorption was measured in octuplicate, and the assays were repeated at least three times. Comparison of expression of antigens between primary bone marrow fibroblasts and the cell line showed that while the positive (ROS 107) and negative (D46) control values were equivalent ($P > 0.05$, Welch's *t* test), the expression of ST3, TIB 103, and Ox-7 was significantly different ($P < 0.0001$).


0.4
0.3
0.2
0.1
0.0
Absorbance
ROS
D46
OX-7
TIB103
ST3
ANTIBODY

LEGEND TO FIGURE 6.

Effect of pre-incubation of the *late-passage* stromal cell layer with various antibodies on subsequent adsorption of clonogenic BNML cells. The results are expressed as in Fig. 3. Each assay was performed in triplicate at least three times on different days. The presence of the noted antibodies did not alter the adhesion from that measured with medium (RPMI) alone (ANOVA, $P > 0.05$).


100
80
60
40
20
0
% OF CONTROL (Non-adherent)
RPMI
D46
TIB
ST3
ANTIBODY

LEGEND TO FIGURE 7.

Comparison of the adhesion of CFU-C to stromal fibroblasts derived from femoral bone marrow and ectopic ossicle marrow. Assays as described for previous figures. Solid bars: adhesion to fibroblasts derived from rat femoral bone marrow; hatched bars: adhesion to fibroblasts derived from ectopic ossicle marrow. The differences between the two cell types are not statistically significant at any time (ANOVA, $P > 0.05$).

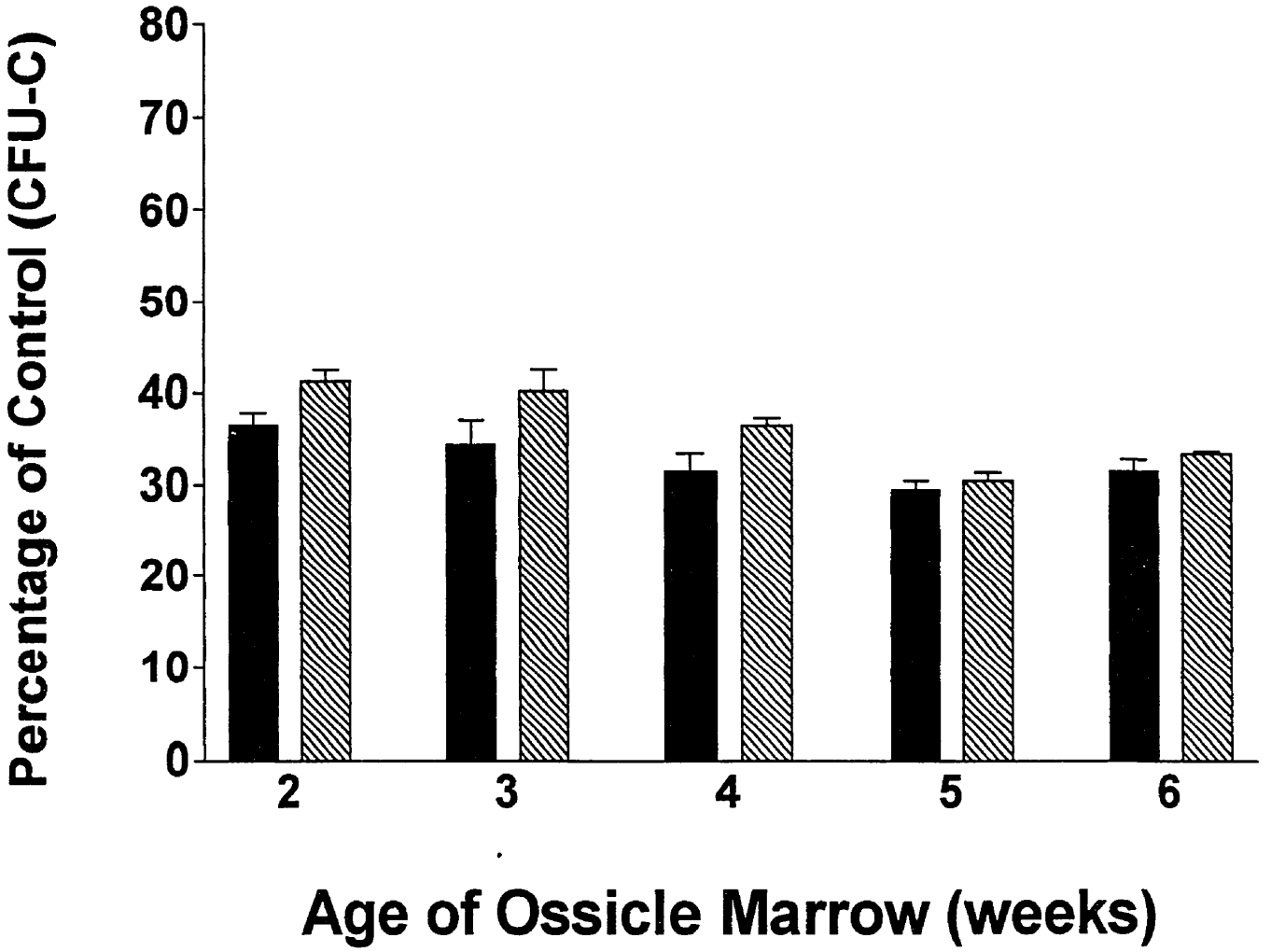

Percentage of Control (CFU-C)
80
70
60
50
40
30
20
10
0
2
3
4
5
6
Age of Ossicle Marrow (weeks)

TABLE 1

ANTIBODY	SPECIFICITY
D46	IgM; negative control
Ros 107	IgG; broad reactivity with rat tissues; positive control
ST3	IgM; predominant population of rat marrow fibroblastoid cells.
ST4	IgG; stains predominant fibroblastoid cell from non-hemopoietic organs.
TIB103 (ATCC)	IgM; Thy-1
Ox-7 (Serotec)	IgG; Thy-1
CRL-1605 (ATCC)	Fibronectin; multiple species
MCA773 (Serotec)	ICAM-1
MCA774 (Serotec)	LFA-1 α chain (CD11)
MCA775 (Serotec)	LFA-1 β chain (CD18)
Y-3P (ATCC HB183)	Rat MHC class II

TABLE 2

Effect of pre-incubation of BNML cells with antibody on subsequent adsorption to the stromal layer.

ANTIBODY:	Colonies in non-adherent fraction*	(%)
BSA**	104.3 ± 3.0	(100)
None (RPMI)	66.0 ± 0.9	(63)
D46	64.7 ± 0.4	(62)
ST3	69.3 ± 0.9	(66)
TIB103	67.8 ± 0.7	(65)
VLA-4	84.8 ± 0.8	(82)

*Colonies/$3x10^2$ BNML cells applied to culture dish; the unmanipulated cell suspension contained 109.8 ± 3.8 clonogenic cells.

** BSA control: colonies recovered after adhesion to dish in the absence of a stromal cell ayer.

Of the antibodies tested, only anti-VLA-4 inhibited adsorption ($P<0.001$); for the others, $P>0.05$.

CHAPTER 4

RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN-2 INDUCES A HEMOPOIETIC MICROENVIRONMENT IN THE RAT THAT SUPPORTS THE GROWTH OF STEM CELLS

PREFACE

In Chapter 3 it was shown that the ST3 epitope, possibly associated with THY-1, could function as an adhesion molecule *in vitro*. In an intact animal, however, hemopoiesis is dynamically regulated by contact between stromal and hemopoietic cells, and by soluble factors, and the production or action of a single regulator may be influenced by other factors acting locally or systemically through complex networks. Therefore, *in vitro* hemopoietic systems can not perfectly represent hemopoiesis *in vivo*. If the ST3 molecule were relevant to *in vivo* marrow function, then it should be expressed during marrow development. To test this hypothesis, I used as a model the *de novo* ossicle induced by subcutaneous implantation of rhBMP, in which the entire process of bone/bone marrow development appeared to occur. To do that, however, I first had to demonstrate that these organoids supported the full range of hemopoiesis.

In addition, included as an Appendix are related data that were not submitted with the published manuscript that formed the major portion of this chapter.

ABSTRACT

In the mammalian bone marrow, stromal components support the growth and differentiation of blood cells. To study this complex system we have used a rat model in which ectopic hemopoietic tissue is induced to form after subcutaneous implantation of recombinant human bone morphogenetic protein (rhBMP-2). We show that this organoid contained clonogenic precursors of both erythroid and myeloid lineages, and progenitors competent to regenerate splenic lymphopoiesis. Furthermore, stem cells derived from ectopic foci conferred both short (30 d) and long-term (> 6 mo) protection *in vivo* against radiation-induced marrow aplasia. Lead shielding of the ectopic marrow *in situ* also permitted endogenous recovery of hemopoiesis after sublethal irradiation. Extending previous observations that most fibroblastoid cells of the marrow stain with the anti-ST3 antibody (but minimally with anti-ST4), whereas those growing from non-hemopoietic tissues react with anti-ST4, we found that analogous cells of the ectopic foci stained predominantly with anti-ST3. The ability to induce formation of a hemopoietic microenvironment from mesenchymal precursors may enable new strategies to be developed for the treatment of primary disorders of stem cells and irreversible stromal injury.

KEY WORDS: bone morphogenetic protein-2, bone marrow stromal cells, bone marrow transplantation, hemopoietic stem cells.

INTRODUCTION.

In adult mammals, the bone marrow is the only organ able to generate a full spectrum of hemopoiesis under normal conditions, and many of the essential positive and negative control elements originate within the stromal compartment. Evidence to support the idea of a regulatory microenvironment derives from two observations: (1) long-term bone marrow cultures continue to produce mature blood cells if a viable stromal layer is present, and (2) hemopoietic tissue develops at extra-medullary sites if marrow stromal cells have been transplanted or induced to differentiate.[1,2,3,4] Further attempts to dissect this complex have used model cell lines and monoclonal antibodies to identify possible subpopulations of specialized function.

In previous reports we described two murine monoclonal antibodies ("ST3" and "ST4") that distinguish between the predominant fibroblastoid cells that grow from rat bone marrow (ST3+/ST4-) and those that originate in certain non-hemopoietic organs (ST4+ cells > ST3+).[5,6,7,8] Although this implies that populations of marrow stromal cells might differentiate into sublineages, proof requires identification of the intermediate steps. One approach would be to examine the process of *de novo* hemopoiesis at an extra-medullary site induced by recombinant human bone morphogenetic protein-2 (rhBMP-2). When placed in the subcutaneous space of a rat, this protein induces local proliferation of mesenchymal precursors that form a focus of endochondral ossification, which then becomes vascularized and eventually encases organized marrow tissue.[9,10,11] Here we show that ectopic ossicles sustain the full range of hemopoiesis, including stem cells with long-term repopulating capacity, and that the fibroblastoid stromal cells they contain express a marrow phenotype.

MATERIALS AND METHODS.

Animals. Inbred male and female Brown Norway (BN) rats age 5-8 weeks were purchased from Charles River Canada (St. Constant, Québec). They were housed in polycarbonate plastic cages and fed standard laboratory chow and water *ad libitum.*

In experiments involving bone marrow transplantation, recipient rats received total body irradiation from a cesium dual-source irradiator (Gammacell 40; Atomic Energy of Canada, Ottawa, Ontario) at a dose rate of 139.4/min and a total dose of 1018 rad. Within 24 hours after TBI the test animals received 2-4x10^6 marrow cells derived from either femoral bones or ectopic ossicles by i.v. injection through the tail vein. Control rats received a sham injection of RPMI 1640 medium. Irradiated and transplanted rats were not raised under germ-free conditions or given other special handling except for acidified drinking water (pH 2.5) containing neomycin (final concentration 0.57 mg/ml), polymyxin B (final concentration 2000 u/ml), and trimethoprim-sulfamethoxazole (final concentration of trimethoprim 110 μg/ml) for 14 days after TBI.

Preparation of ectopic bony ossicles. Gelatin capsules containing recombinant human bone morphogenetic protein-2 (rhBMP-2; Genetics Institute, Cambridge, MA) attached to a carrier of demineralized rat bone powder were prepared as described. [11] Rats were anesthetized by abdominal injection of ketamine hydrochloride (100 mg/kg), and under aseptic conditions a 1 cm midline incision was made in the skin of the upper abdomen or lower thorax through which a subcutaneous pocket was formed bilaterally by blunt dissection. A capsule containing BMP-2 (5-15 μg/capsule) was placed within the pocket, and the incision was closed with silk sutures. In each 5-6 week old BN rat, depending on the design of the particular experiment, 2-4 capsules were implanted. The day of implantation was designated day 0; on each of weeks of 2, 3, 4, 5, 6, and 8, two to three experimental rats were killed, and the bony ossicles were recovered and prepared for subsequent analysis.

Preparation of cell suspensions. Cell suspensions were prepared from normal bone and ossicle marrow by flushing the femoral shafts or fractured ossicle with cold medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100

U/ml penicillin, and 100 μg/ml streptomycin). Cell suspensions of spleen and thymus were prepared by mincing the tissue in RPMI medium, and dispersing the aggregates through a fine mesh sieve. Blood samples from rats were withdrawn from the heart and placed into EDTA-containing "microtainer" tubes (Becton, Dickinson, Vacutainer).

Skin and lung fibroblastoid cells were obtained by removing the tissue, mincing it into small pieces, incubating with 200 U/ml collagenase (Sigma, Type II; Cat. No. C6885) in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone, for 2 h at 37° C in a shaking incubator, resuspending every 30 min. Finally, the mixture was incubated with 0.25% trypsin for 1 hour at 37° C, and filtered through a fine mesh sieve. After centrifugation over a Ficoll-Hypaque barrier, nucleated cells were collected from the interface and washed three times with regular medium. All cell suspensions were centrifuged at 1200 rpm for 5 min, and the cell pellets were resuspended in the appropriate medium, depending on subsequent use. All cell counts were performed using a hemocytometer.

Preparation of tissue sections and cytospin slides. The ossicles were removed, fixed in Zenker's solution, and embedded in paraffin by standard methods. Serial sections (5 μM) were cut, mounted on microscope slides, and stained as described.

Cells in suspension ($1.5\text{-}4.5 \times 10^5$) were applied to microscope slides using a cytocentrifuge (Cytospin 2, Shandon Southern Products, Ltd.), and were either fixed in 100% methanol containing 0.3% H_2O_2 for 30 min at room temperature, or used after air drying. Staining was performed as previously described [5] by the Giemsa method, or for detection of endogenous peroxidase or immunoperoxidase. Antisera to von Willebrand factor (rabbit anti-human factor VIII-related antigen; Dakopatts; #A082, Dimension Laboratories, Mississauga, Ontario) was absorbed twice with rat erythrocytes and spleen cells prior to further use.

Clonogenic assay for hemopoietic precursors. Assay for colony-forming units (CFU-G/M and CFU-E) of the rat has been described elsewhere.[6] Briefly, a cell suspension was prepared from normal bone or ossicle marrow in RPMI 1640 medium containing 10% FCS, and mixed using a syringe with a 21 gauge needle. One ml of the culture system consisted of the following:

- Iscove's Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 30% FCS, 1% BSA;

- 5% spleen cell-conditioned medium (SCCM) for assay of CFU-G/M; or

- 1 unit/ml erythropoietin (step III, purified from sheep, Connaught Laboratories, Willowdale, Ontario; No. 1501-5-7) for assay of CFU-E;

- $5x10^{-5}$ M 2-mercaptoethanol; 0.8% methyl cellulose (4000 centipoise; Fisher Scientific #M-352); and $1.5x10^5$/ml nucleated marrow cells.

The mixture was added to a 35 mm culture dish and incubated at 37°C in an atmosphere containing air/5% CO_2. Erythroid colonies (CFU-E) were scored on day 2, and myeloid colonies (CFU-G/M) on day 7 of culture, according to standard criteria using an inverted microscope.

The SCCM was prepared by culture of nucleated spleen cells ($1x10^6$/ml) in RPMI 1640 medium containing 10% FCS, 1% BSA, 2 mM L-glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml phytohemagglutinin (PHA-L Pure E.Y. Labs, San Mateo, CA). After 4-5 days the culture supernatant was harvested, centrifuged at 1200 rpm for 30 min, and stored at -20° C. The concentration of SCCM used was that which caused maximal stimulation of colony formation in preliminary assays.

Stromal fibroblastoid cell culture. Nucleated cell suspensions ($2-20x10^5$) were obtained from different tissues (femoral bone marrow, ossicle marrow, spleen, skin, and lung) as described above, distributed into 75 cm^2 flasks or single-well chamber slides, and cultured in RPMI 1640 medium containing 10% FCS, 5% rat serum, 1% BSA, 2 mM L-glutamine, 100 u/ml penicillin, and 100 μg/ml streptomycin for 2 weeks without medium change, and then continued for another 1-2 weeks with half-medium changes weekly. For cultures of lung tissue, the media also contained 2.5 μg/ml fungizone. To generate homogeneous stromal monolayers, the adherent cells were detached by incubating with 0.05% trypsin-PBS (Gibco), recultured, and passaged at least four times. The hemopoietic cells were depleted by three cycles of treatment with mycophenolic acid (Sigma #M5255) at a final concentration of 5 μg/ml; macrophages were depleted by antibody-dependent complement-mediated cytotoxicity (BN35 antibody; murine monoclonal to rat myeloid cells, including CFU-G/M) as previously described.[6] After

4-8 passages, fibroblastoid cells were removed from tissue culture flasks by a 5-8 min incubation in trypsin-PBS at 37° C. After two washes with regular medium, cells were distributed into either 96-well culture dishes and/or 4-well chamber slides (Lab-Tek #177437) at $1x10^4$ cells per well, and cultured for 3-5 days. Subsequent analysis was either by quantitative ELISA assay (dishes) or immunohistological staining (chamber slides).

Clonogenic fibroblastoid cells (CFU-F) were measured according to the method of Castro-Malaspina, as modified by McIntyre and Bjornson.[12] Briefly, normal bone or ectopic ossicle marrow ($2x10^5$ nucleated cells) was cultured in single well chamber slides (Lab-Tek single chamber tissue culture slides, #177410; Miles Scientific, Rexdale, Ontario) in 1.5 ml RPMI 1640 medium supplemented with 10% FCS, 5% rat serum, 1% BSA, 2 mM L-glutamine, 100 u/ml penicillin, and 100 μg/ml streptomycin. Duplicate or triplicate chamber slides were incubated in a 37° C humidified incubator with an atmosphere of 5% CO_2/95% air for 10-14 days. Colonies were enumerated by direct visualization under the light microscope; a colony was defined as an aggregate of >50 cells.

Enzyme linked immunosorbant assay (ELISA) and immunohistochemical staining. Cells prepared as described above were fixed in 0.05% glutaraldehyde in PBS (pH 7.4) for 10 min. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 for 30 minutes. After rinsing 3x with PBS, the cells were incubated for 20 minutes in 0.1% gelatin/PBS, and again rinsed 3x with PBS prior to a 30 min incubation with the primary murine antibody at the appropriate dilution. After rinsing with 0.1% BSA/PBS, another 15 min incubation with 0.1% gelatin/PBS, and another 3 rinses with PBS, the preparation was incubated for 30 min with peroxidase-conjugated goat anti-mouse antiserum (anti-IgG + IgM heavy and light chains; #041809 Kirkegaard Perry through Mandel Scientific Co., Rockwood, Ontario; diluted 1:100 for chamber slide staining or 1:500 for ELISA in 0.1% BSA/PBS). After rinsing, the substrate reaction was as follows: (1) Chamber slide histology: AEC (0.2 mg/ml 3-amino-9-ethylcarbazole, Aldrich Chemical Co.) in the AEC buffer (0.10 M acetate; pH 5.1) was added to each slide for 10-15 minutes at room temperature, and then rinsed with PBS, counterstained

with Mayer's hematoxylin for 30 min, rinsed with warm tap water, and mounted in glycerol/PBS. (2) ELISA assay: Preparations were incubated with the peroxidase substrate (containing 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) tablets (Sigma Chemical Co. A-4798 #112H8903) for 30-45 minutes, and the absorbance was measured with a microplate ELISA reader. Negative control antibodies included subclass specific murine monoclonals of irrelevant specificity; positive controls included uncharacterized murine antibodies selected for broad reactivity with rat tissues.

Verification of bone marrow chimerism after transplantation. Female animals were transplanted with male-derived femoral or ossicle marrow induced with rhBMP-2, and sacrificed at times indicated in the "Results". Engraftment of the recipient was verified using a rat Y-chromosome specific probe described elsewhere (manuscript submitted). Briefly, rat genomic DNA was extracted from nucleated cells of marrow and spleen by standard methods. For Southern blots, the EcoR1 (Phamacia Biotech, #270854-18) digested genomic DNA (3-5 μg) was electrophoresed on a 0.8% agarose gel and transferred to a "Gene Screen Plus" membrane (Dupont, #NEF-976). The prehybridization (4-6 hours) and hybridization (12-16 hours) was performed at 42° C; the hybridization solution contained the following: 50% deionized formamide, 1x Denhardt's solution, 5x SSPE (SSPE: 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA), 10% dextran sulphate, 100 μg/ml denatured salmon sperm DNA, $1x10^6$ cpm/ml ^{32}P-α-dATP-labelled probe (described below), and 1% SDS. Finally, the membrane was washed twice in 2x SSC/0.1% SDS (SSC: 0.15 M NaCl, 0.01 M Na citrate), 20 minutes each at room temperature, once in 0.1 x SSC/0.1% SDS for 30 minutes at 65° C, and autoradiographed for 3-5 days.

The rat Y-specific probe was derived as follows. Briefly, rat genomic DNA was amplified by PCR using two synthetic primers based on published sequences from the murine sex determining region (*sry*) of the Y chromosome (5'AGATCTTGATTTTTAGTGTTC 3' and 5' TGCAGCTCTACTCCAGTCTTG 3').[13] A DNA fragment of 459 bp was shown to be amplified in samples of male but not female tissues. Subsequently, this PCR product was cloned by using a "TA" cloning vector (Invitrogen, San Diego, CA, K2000-01), and competent *Blue-XL-1 (SK^+) E coli.*

The plasmid DNA containing the insert was extracted from the successfully transformed E coli and digested with *EcoR1*; an *EcoR1* fragment of 270 bp was isolated, and labelled with α^{32}P-dATP (3000 Ci/mM, Dupont, #NEG-012H) by the random priming technique.

RESULTS.

Ectopic marrow contains erythroid, myeloid, and megakaryocyte lineages. Within 2-3 wk after subcutaneous implantation of a gelatin capsule containing rhBMP-2, a well vascularized bony nodule forms at the site. Over the next 2-4 wk, tissue reminiscent of active hemopoietic marrow appears within the remodelling bone.[10,11] By 5 wk the new tissue is richly cellular and contains morphologically-diverse cells within inter-sinusoidal spaces (Fig. 1). Cytospin preparations made from suspensions of ossicle marrow confirmed the presence myeloperoxidase-containing cells at various stages of maturity, structures resembling erythroblastic islands in which a central macrophage was surrounded by maturing normoblasts, and megakaryocyte-like cells containing von Willebrand antigen (Figs. 1 & 2).

Clonogenic hemopoietic precursors are present in ectopic marrow. To determine if committed blood cell precursors expand in ossicle tissue, clonogenic assays were performed periodically after implantation of the BMP-containing capsules. The time course of detection of myeloid (CFU-C) and late erythroid (CFU-E) colonies is illustrated in Fig. 3. By 5-6 weeks, the frequency of both myeloid and erythroid colonies had reached the level observed in normal femoral bone marrow. Typical of rat marrow cultured under these conditions, most of the CFU-C were composed of macrophages, but in both femoral marrow and mature ossicles, granulocyte-containing colonies (CFU-G/M and CFU-G) comprised 8-12% of the total (not shown). In control experiments, the number of myeloid and erythroid colonies in peripheral blood did not rise above baseline, indicating that BMP did not simply mobilize immature elements into the circulation.

Cells capable of radioprotection are present in the ossicles. The detection of maturing blood cells, committed precursors, or even CFU-S in the ectopic foci would not

necessarily prove that pluripotent stem cells were present. To determine if the ectopic nodules contained cells capable of repopulating the bone marrow, cell suspensions were prepared from the ossicles and infused into lethally irradiated rats. Whereas all of the non-transplanted control animals had died by 15 days after irradiation, approximately 50% of those reconstituted with either ossicle or femoral marrow survived for longer than 4 weeks (Fig. 4), and over 30% survived longer than 6 months.

Another characteristic of stem cells is their ability to migrate into the circulation and seed a receptive stroma. To test whether the reconstituting cells are fixed within the bony nodules, or have access to the circulation, two mature ossicles in each rat were covered with a lead shield prior to irradiation. As shown in Table 1, 3/6 animals survived longer than three months, in contrast to 0/6 that had not been shielded, supporting the notion that the tissue that formed in response to BMP-2 contained competent hemopoietic stem cells which could exit the marrow and establish hemopoiesis at a distant site.

Ossicle-derived cells include progenitors with long-term repopulating ability. Progenitor cells capable of short-term radioprotection may differ from those giving rise to long-term repopulation of the bone marrow.[14] To determine whether true stem cells were present, female rats were transplanted with male-derived ossicle marrow to enable detection of donor cells using a male-specific cDNA probe. As shown in.Fig. 5, both the spleen and marrow of female animals that had survived transplantation for over 6 months contained male-derived cells. Lacking a female-specific marker, however, it is possible that they had remained chimeric.

Ossicle fibroblastoid stromal cells express a marrow phenotype. If the mesenchymal cell reaction induced by rhBMP-2 eventually reconstructs a functioning bone marrow architecture at the site of implantation, then it follows that a functioning hemopoietic microenvironment also had formed. Measurement of clonogenic fibroblastoid cells (CFU-F) in developing ossicles indicated that prior to two weeks after implantation the frequency was higher than what was measured from femoral marrow, but between 2-3 weeks it decreased to slightly below the femoral CFU-F value (Fig. 6). Quantification of ST3 and ST4 antigens on fibroblastoid cells grown from the ectopic

marrow showed that the ST3 type predominated, but a minor subpopulation of ST4+ cells (< 20%) was present at 2-4 wk, which had almost completely disappeared by 6 wk (Fig. 7). In contrast, adherent cells grown from skin taken from the area around the implantation site expressed both ST3 and ST4 antigens.

DISCUSSION.

The induction of active hemopoiesis at the ectopic site implies that the mesenchymal cell reaction stimulated by rhBMP caused differentiation of local stromal cell precursors into a microenvironment permissive to the homing and subsequent differentiation of hemopoietic stem cells. Since endogenous repopulation occurred in irradiated animals which had been shielded over the nodule, stem cells appear to be maintained and recirculate *in vivo*. Using two monoclonal antibody markers previously shown to be expressed differentially on fibroblastoid cells of different tissues, we showed that the phenotype of the stromal cells that grew from the recovered ossicles was similar to that of femoral bone marrow. The observation that the ST3 marker was expressed on most fibroblastoid cells of the ectopic marrow raises the question of what role, if any, it might play in stromal function. Our preliminary data indicate that the adhesion of committed myeloid precursors to marrow fibroblasts expressing the ST3 antigen can be partially blocked by the ST3 monoclonal antibody, which biochemical analysis suggests is directed to an epitope carried on the Thy-1 molecule (manuscript in preparation).

Other researchers have reported that hemopoietic tissue can be induced to form at extramedullary sites. The classic studies reported by Friedenstein in 1974 showed that marrow fibroblasts implanted beneath the renal capsule of a guinea pig induced hemopoietic tissue, and established the notion that immature blood cells can form foci outside the marrow if a permissive microenvironment is supplied.[15] Later work by Knospe and collaborators demonstrated that trilineal hemopoiesis can develop on an artificial membrane if a stromal layer had been grown on it *in vitro* before it was implanted into the peritoneal cavity of a mouse.[4] However, it was Reddi and Huggins who clearly showed that demineralized bone matrix could induce mesenchymal cells to

differentiate into a bony nodule that would remodel and support marrow tissue.[10] Studying bony ossicles induced by semi-purified mixtures of BMP's, others showed that both myeloid precursors (CFU-GM) and multipotent progenitors (CFU-S) could be present in ectopic foci.[16,17] The possibility that true stem cells might develop in similar foci was suggested by the observation that cells from intramuscular ossicles induced by tumour-derived epithelia could rescue other lethally-irradiated animals.[18] However, the active components of these substances were not identified, and the time course of evolution of the marrow tissue was much longer than what we have observed with rhBMP-2.

In addition to what is sometimes observed in the liver and spleen during compromised bone marrow function (e.g., myelofibrosis or chronic hemolytic anemia), extra-medullary hemopoiesis occurs also in pathological conditions. Extra-adrenal myolipoma can contain cells of all hemopoietic lineages, and are thought to arise either from embryonic rests of stem cells in the adrenal, or metaplasia from resident mesenchymal/vascular cells. Notably, these tumours do not form bone.[19] Other non-adipose tumors which contain bony spicules can occur in the retroperitoneal or mediastinal spaces.[20] Thus, hemopoietic tissue can grow outside of the bone marrow if local conditions are permissive.

Insight into mechanisms for how bone and marrow develop *de novo* in the adult mammal have come from recent studies of BMP's in both embryonic and adult systems. Individual BMP's have been localized by *in situ* hybridization to sites undergoing skeletal formation in mouse embryos.[25,27,21] While each BMP studied to date has a distinct pattern of expression, the osteogenic BMP's all appear to be involved in the differentiation of cartilage and bone cells from mesenchymal cell progenitors. *In vitro*, limb bud cells derived from embryonic mice have been shown to be highly responsive to BMPs, with exposure to BMP-2, BMP-4, BMP-7 or osteogenin resulting in the differentiation of these cells into chondroblasts and osteoblasts.[22,23,24]

The recombinant-derived product of the human BMP-2 gene used in these studies is one of several members of the "decapentaplegic" subfamily within the TGF-β gene superfamily. Of recognized importance in insect embryogenesis, these genes also appear

in different subpopulations of mammalian mesenchymal cells during development of the skeletal system.[25] In the adult rodent, histologic studies have shown that fibroblastoid precursors gradually transform into chondrocytes under the influence of BMP's.[26] However, this protein appears also in organs other than bone, including the heart and hair follicles, in a pattern different from that of TGF-β1, -2, or -3.[27] BMPs also have been shown to be reparative agents in adult animals. Treatment of critical size bone defects in rats, sheep, rabbits, and dogs, with BMP results in healing of the defect through the process of endochondral bone formation. Return of both form and function, including active marrow, are found at the affected site.[28,29] *In vitro*, osteoprogenitor cell lines isolated from adult bone marrow stroma and adult bone respond to BMP treatment by differentiating along an osteoblast pathway.[30,31,32] Mature bone cells also have been reported to respond to BMP treatment by increasing the synthesis of extracellular matrix proteins such as collagen type I and osteocalcin, which are abundant proteins in bone matrix.[33] These data suggest that BMP-2 is able to act at the level of osteoprogenitor cells to induce their differentiation into osteoblast-like progeny.

Our experiments show that the "BMP-2 cascade" generates an ectopic bone marrow that contains the stromal elements necessary to support growth and differentiation of hemopoietic stem cells. Such a model, based upon the unusual ability of a single protein to induce reconstruction of an organ in an adult mammal, should allow one to define the steps between a mesenchymal precursor cell and a functioning hemopoietic microenvironment. Furthermore, the potential to produce active bone marrow outside the medullary space could be desirable in certain clinical conditions. Although the distribution of subcutaneous fat, or other differences between rodents and primates, might adversely influence the response of mesenchymal progenitors to BMP-2, autologous transplantation from a *de novo* source might circumvent some of the problems encountered in transplantation of individuals with leukemia or irreversible stromal injury. Finally, the ability to assemble a stable, vascularized organoid under controlled conditions could add another dimension to strategies for gene therapy of disease.

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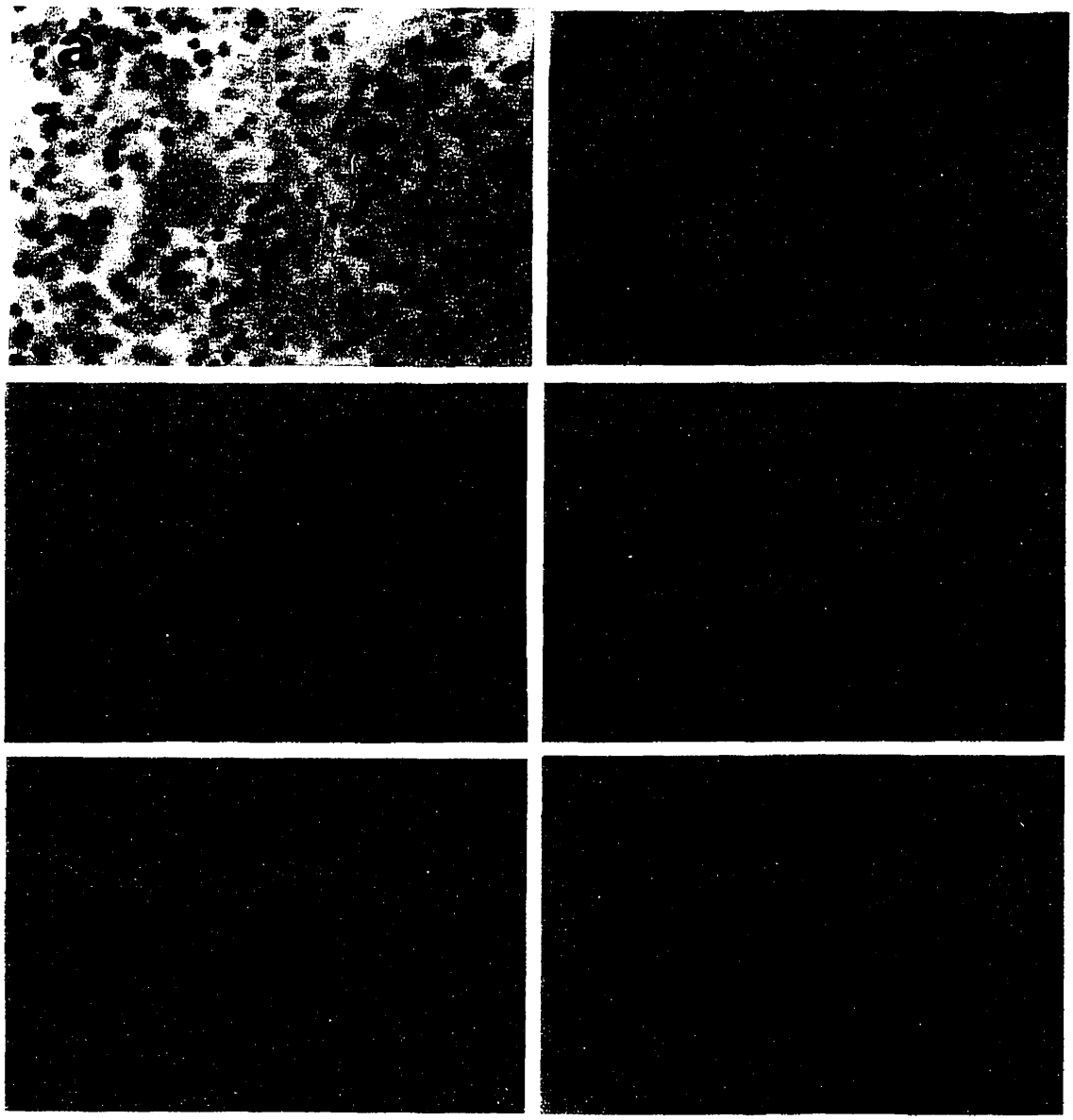
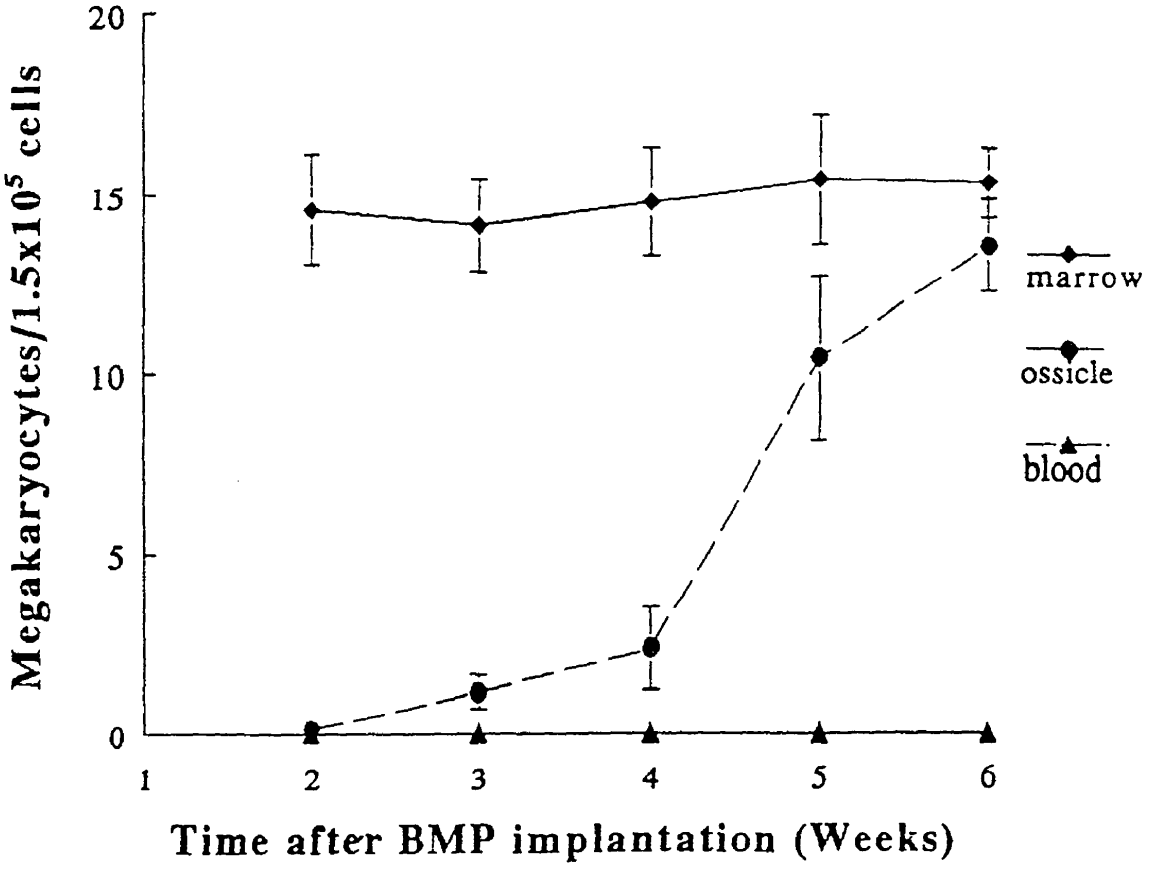
LEGEND TO FIGURE 1

Appearance of hemopoietic cells formed within the bony ossicle. a: Representative histologic section through a bony ossicle removed 5 wk after implantation of rhBMP-2, processed by routine methods, and stained by the periodic acid-Schiff reaction to better visualize megakaryocytes; 100x magnification. b: Cytospin preparation of cell suspension from mature ossicle stained with Jenner-Giemsa showing maturing erythroid and myeloid cells; magnification 150x. c: Cytospin preparation, prepared as in (b), showing an erythroblastic island; magnification 150x. d: Cytospin preparation stained for endogenous myeloperoxidase activity; magnification 150x. e: Cytospin preparation, immunoperoxidase stain with polyclonal antiserum to von Willebrand factor; magnification 100x. f: Cytospin preparation, immunostained with antisera to von Willebrand factor; magnification 400x.


a)

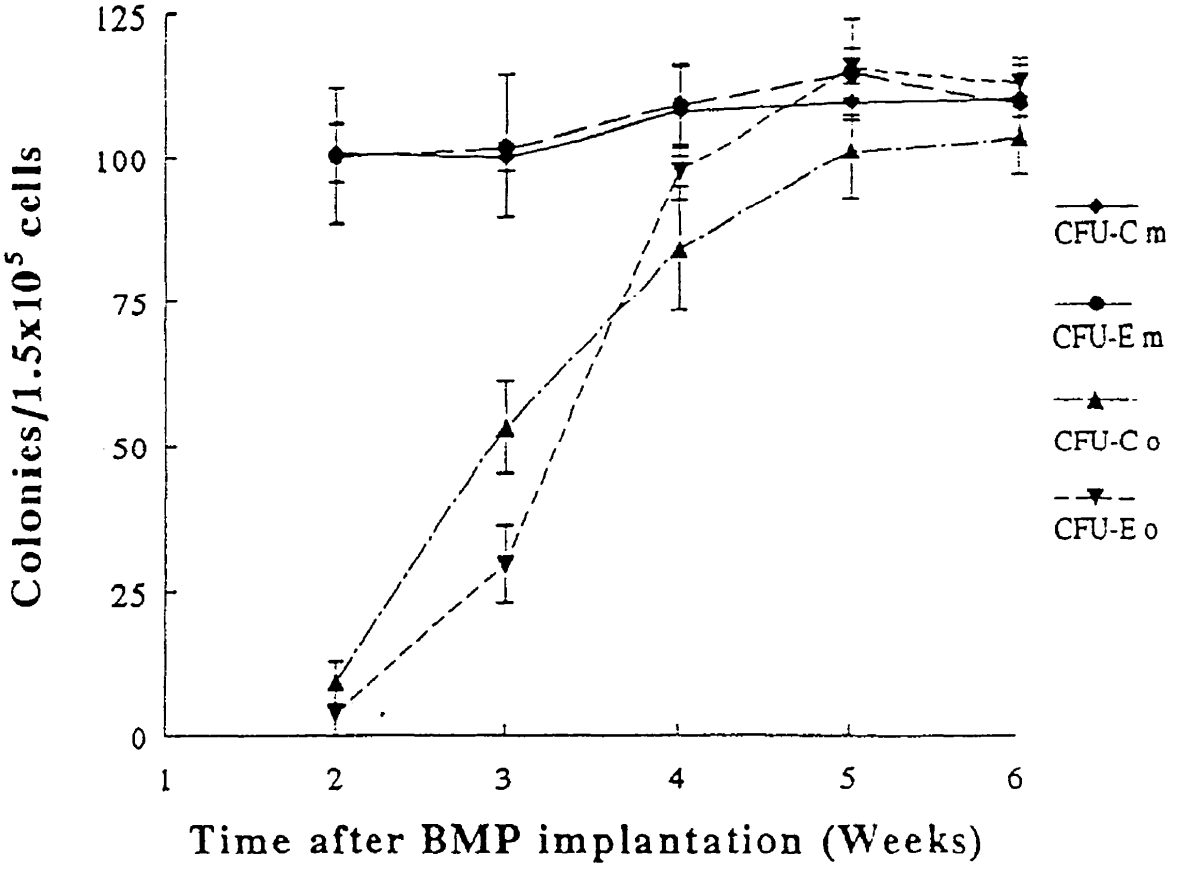
LEGEND TO FIGURE 2

Time course of appearance of megakaryocytes in ectopic marrow. Cell suspensions were prepared from ossicle marrow at the stated time, cytospin preparations were prepared and immunostained with antisera to von Willebrand factor. Large reactive cells with the appearance of megakaryocytes were enumerated on duplicate slides. Each time point on the graph represents the mean $\pm$ 1 SD of results from three ossicles from three rats. In addition, the entire sequence for these experiments, and those in subsequent figures, was performed twice separated by a time interval of three months. Diamonds (♦) denote bone marrow control slides; triangles (▲), peripheral blood controls; and dots (●), ossicle marrow, all from the same rats.


Megakaryocytes/1.5x10^5 cells
20
15
10
5
0
1
2
3
4
5
6
Time after BMP implantation (Weeks)
marrow
ossicle
blood

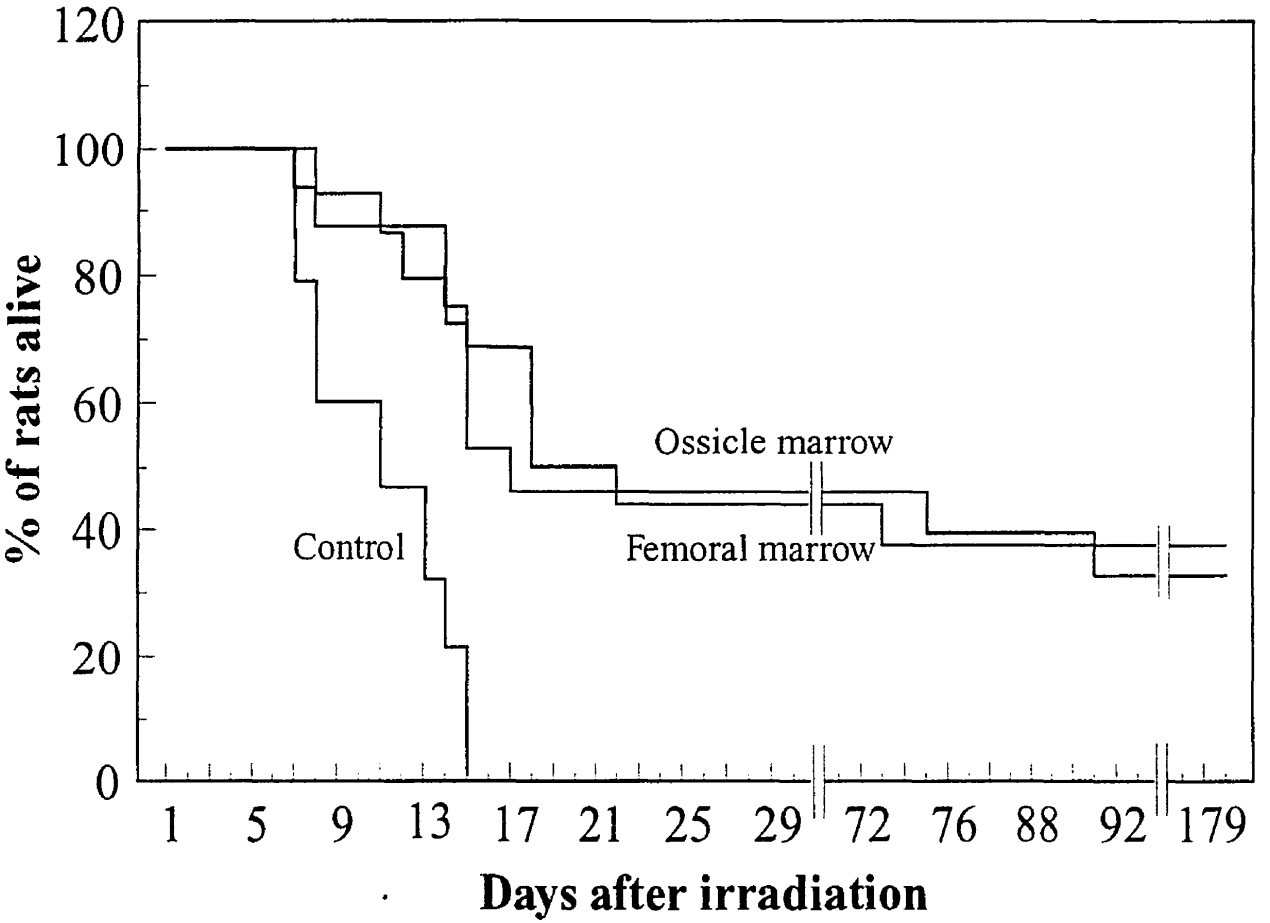
LEGEND TO FIGURE 3

Frequency of committed myeloid and erythroid precursors in ectopic ossicles. Capsules containing BMP-2 were implanted, and the resulting bony ossicles allowed to develop. After they were recovered, cell suspensions were used to measure (in duplicate assays) the presence of granulocyte/macrophage and erythroid colony-forming units (CFU-C and CFU-E) as described in the "Methods". CFU-Cm (diamonds: ◆) and -Em (dots: ●) refer to colonies from femoral marrow, and CFU-Co (up-triangle: ▲) and -Eo (down-triangle: ▼) refer to ossicle marrow. At each time point, cells from femoral marrow, a single ossicle, and peripheral blood were recovered from the same rat, and the CFU-C assay was performed. Each data point (± SD) represents the mean of three rats. The entire sequence was performed twice and the results combined in a single graph.


125
100
75
50
25
0
1
2
3
4
5
6
Colonies/1.5x10^5 cells
Time after BMP implantation (Weeks)
CFU-C m
CFU-E m
CFU-C o
CFU-E o

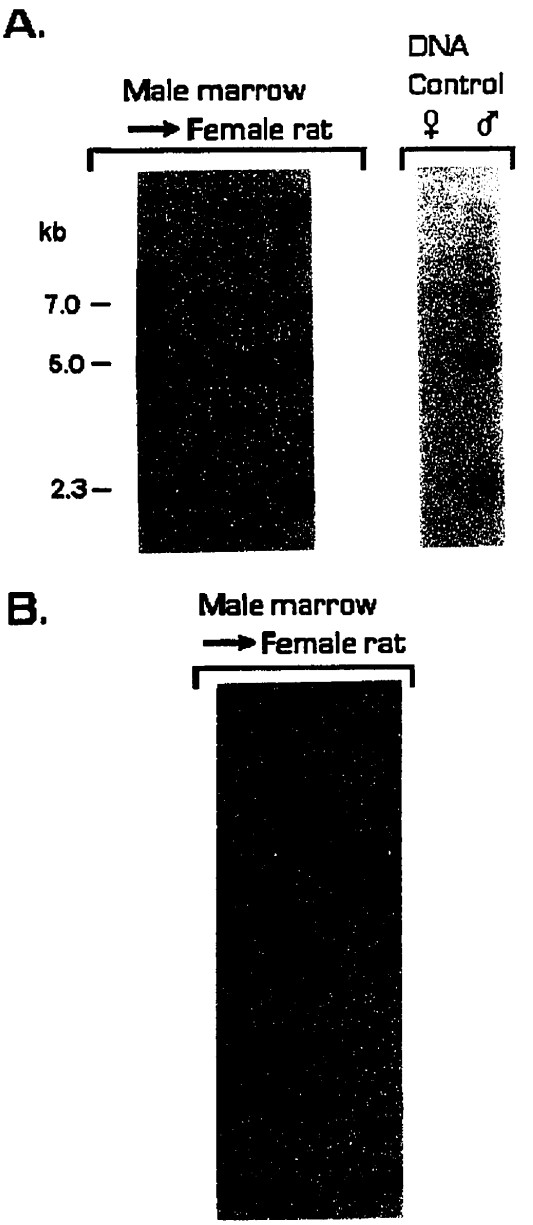
LEGEND TO FIGURE 4

Survival of rats transplanted with cells from femoral bone marrow or ectopic ossicles. Bony nodules were induced as described, two of which were recovered from a single rat at either 5 or 8 weeks after implantation; cell suspensions were prepared and infused into irradiated hosts. The entire sequence was performed twice; because the results were essentially identical with 5 or 8 week-old ossicles, the results were pooled. Total number of animals in each group: non-transplanted controls = 15; transplanted with femoral bone marrow = 16; transplanted with cells from ossicles = 15.


120
100
80
60
40
20
0
% of rats alive
Ossicle marrow
Femoral marrow
Control
1
5
9
13
17
21
25
29
72
76
88
92
179
Days after irradiation

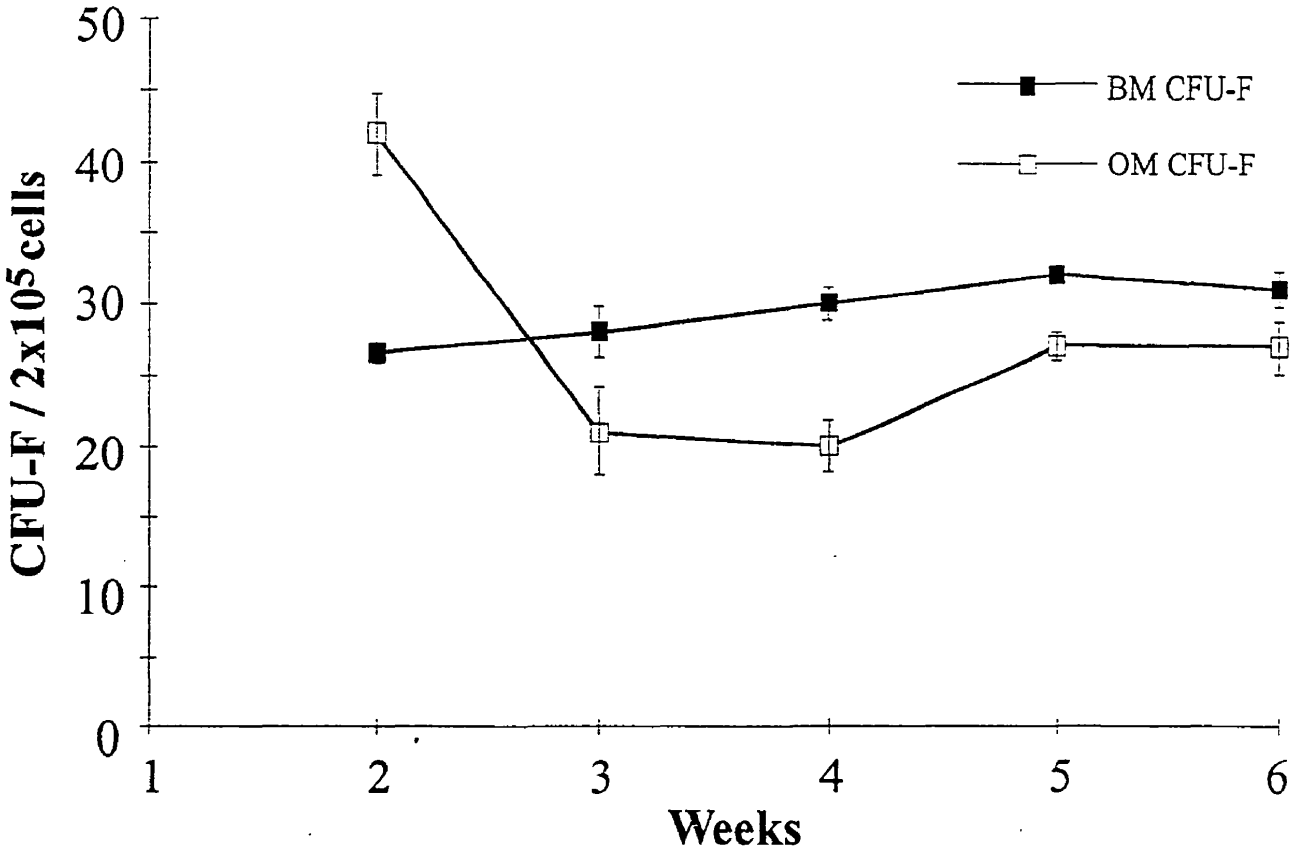
LEGEND TO FIGURE 5

Detection of male DNA in chimeric female rats. Six months after infusion of male ossicle marrow cells into irradiated female rats, four animals were sacrificed, and cells from the bone marrow and spleen were recovered for DNA analysis by Southern blotting using a probe specific for the Y-chromosome. Panel A: Bone marrow from four female rats which had received male ossicle marrow; DNA control: bone marrow DNA from male and female rats that had not received foreign cells. Panel B: Spleen cells from four female rats transplanted with male ossicle marrow.


A.
DNA
Control
♀ ♂
Male marrow
→ Female rat
kb
7.0 —
5.0 —
2.3 —
B.
Male marrow
→ Female rat

LEGEND TO FIGURE 6

Comparison of clonogenic fibroblastoid cells grown from femoral and ossicle marrow. Solid squares (■): BM CFU-F, fibroblastoid colony-forming units from femoral bone marrow. Open squares (□): OM CFU-F, fibroblastoid colony-forming units from ossicle marrow.


50
40
30
20
10
0
CFU-F / 2x10^5 cells
1
2
3
4
5
6
Weeks
BM CFU-F
OM CFU-F

LEGEND TO FIGURE 7

Quantification of fibroblastoid cells expressing the ST3 or ST4 antigen. Fibroblastoid cells from the noted organs were cultured, and the ST3 or ST4 antigens were quantified by ELISA as described in the "Methods". Hatched bars: assay of ST3 antigen expression, open bars: assay of ST4 expression. The 100% control value ("BM" or "LUNG") for each set of experiments was the result obtained for bone marrow-derived (for assays of ST3) or lung-derived (for ST4) cells, treated identically to cells from the ossicle. The ossicle cells were harvested at the times noted, cultured, and assayed. Each value shown is the mean of eight replicate wells; the entire sequence was repeated at least three times, with ossicles from two or three rats being analyzed at each time point. Error bars show mean $\pm$ SEM.

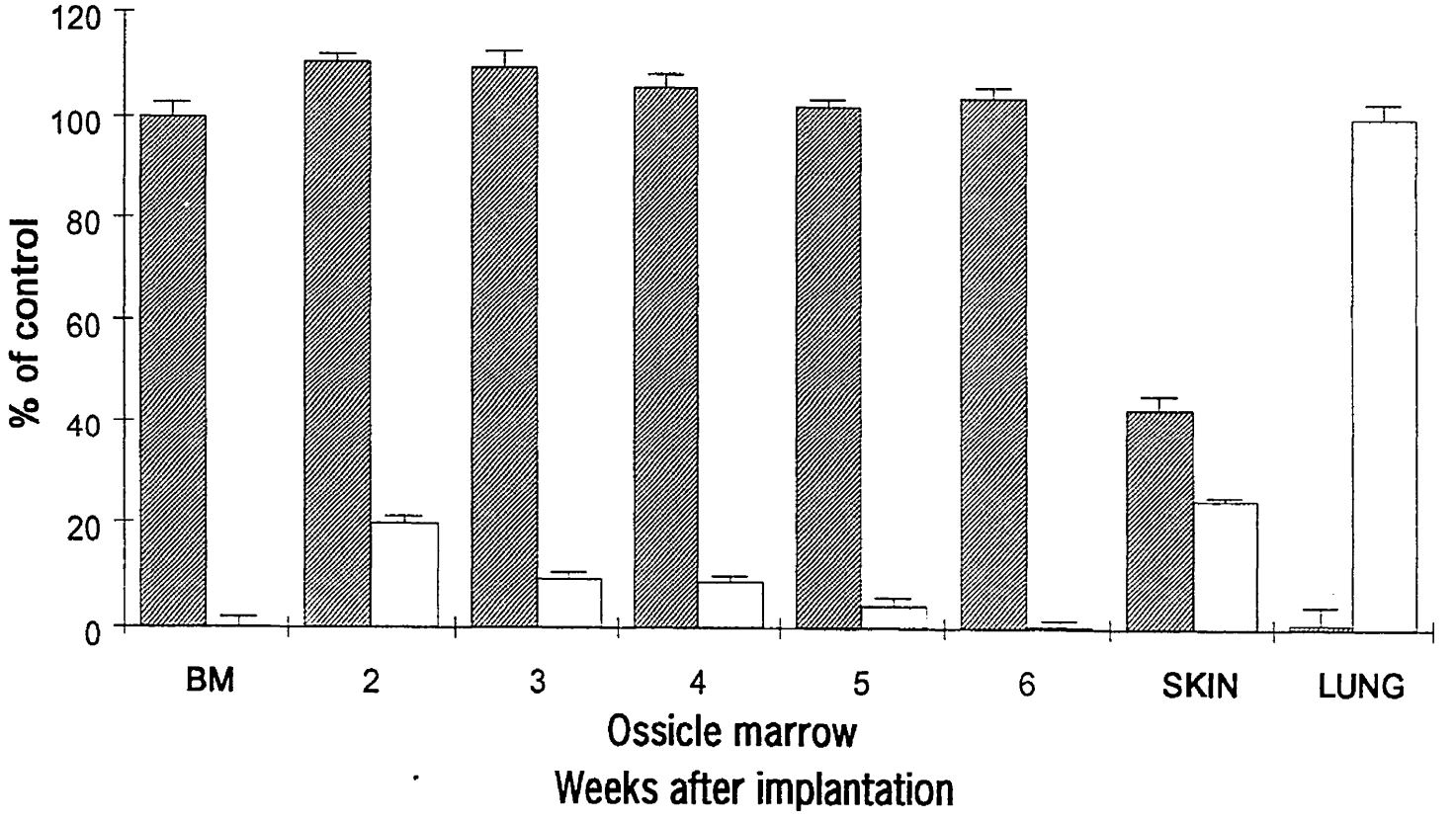

120
100
80
60
40
20
0
% of control
BM
2
3
4
5
6
SKIN
LUNG
Ossicle marrow
Weeks after implantation

TABLE 1

EFFECT OF SHIELDING OSSICLE FROM IRRADIATION ON SUBSEQUENT SURVIVAL OF THE ANIMAL.

EXPERIMENT	No. ANIMALS	SURVIVAL (days)
1	3	47, 48, >90
2	3	64, >90, >90

Ossicle-bearing animals were irradiated as if they were to be transplanted with bone marrow, but instead the ossicles were protected by a lead disk. Subsequently, they were treated identically to the transplanted animals. All six ossicle-bearing control animals which had not been shielded died between day 15-18.

APPENDIX

OTHER CELLS IN THE DEVELOPING OSSICLE

Although the focus of this work was not to define all stages of the developing ossicle induced by rhBMP, we performed preliminary experiments to examine different cells types. This Appendix shows examples of the mesenchymal cell reaction in the early period, and shows how macrophages also may be an important component. Also, we performed pilot experiments to test the feasibility of the using ectopic ossicles to study mechanisms of metastatic spread to the bone marrow.

METHODS (not included in other sections).

Antibodies. Monoclonal antibodies used were the following: BN35, Ros73 (negative control), Ros107 (positive control). The BN35 antibody has been shown to stain myeloid precursors and mature macrophages. Antibodies were diluted in RPMI 1640 medium and used at predetermined optimal dilutions.

Tissue sections and cytospin slides preparations. The ossicles were removed, fixed in either Zenker's solution or 80% ethanol and embedded in paraffin by standard methods. Serial sections (5μM) were cut, mounted on microscope slides and stained either hematoxylin-eosin (H-E) or Crocott stain. Suspension cells (1.5-4.5x10^5) were fixed onto a microscope slide using a cytocentrifuge.

RESULTS.

Histology of the early stage of the developing ossicle. Two weeks after implantation of rhBMP, fibroblasts, undifferentiated mesenchymal cells, and chondroblasts predominated (Fig. 1A-C). At this stage, hematopoietic precursor cells were not recognized by morphologic analysis (a CD34 equivalent was not available for

the rat to perform flow cytometry). Between 3-4 weeks, the cellularity increased in the space between remodelling bones (Fig. 1D). The typical marrow architecture was formed after 5 weeks and was sustained past 8 weeks (Fig. 1E). In addition, frequent multinucleated giant cells were observed in cytospin preparations from 3 week ossicles (Fig. 1F).

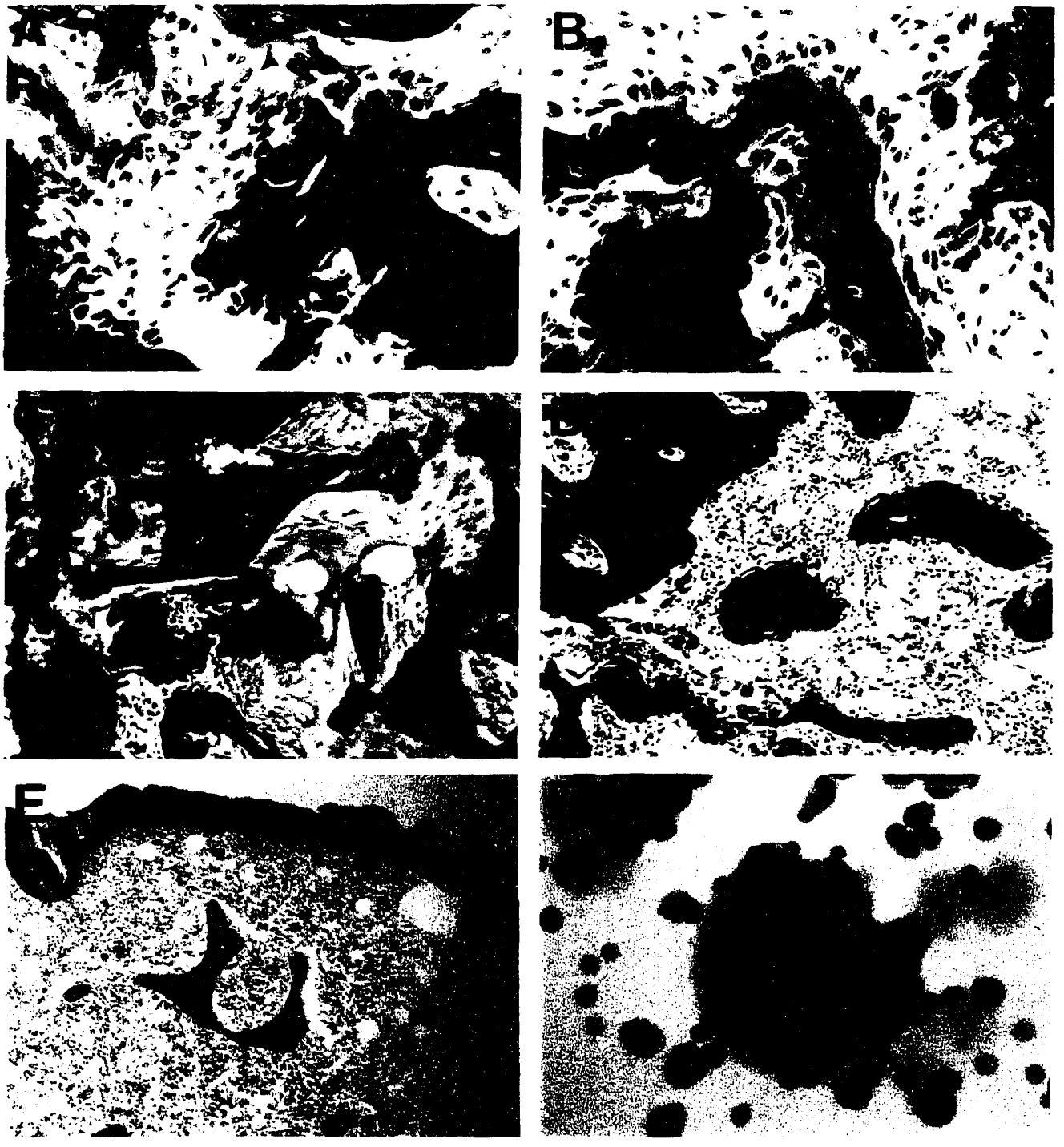
Macrophage in the developmental ossicle. The BN35 antibody identifies rat macrophages. The frequency of $BN35^+$ cells was maximal at 2-3 weeks after BMP implantation, but by 5-6 weeks had decreased to baseline (Fig. 2).

Colony stimulating activity of stromal fibroblastoid cells from ossicles. One of the potential functions of fibroblastoid cells is the production of cytokines. The release of colony stimulating activity for CFU-C into the culture supernatant of fibroblastoid cells derived from 2-6 weeks ossicles (Fig. 3.) was measured, and shown to be equivalent to that released from equivalent cells obtained from authentic marrow.

Invasion of BNML cells into the ossicle marrow. For future studies on the entry of leukemic cells into the bone marrow, pilot studies were performed to assess whether BNML cells would home to and grow within ossicles. The experiments shown in Fig. 4 shows that they do.

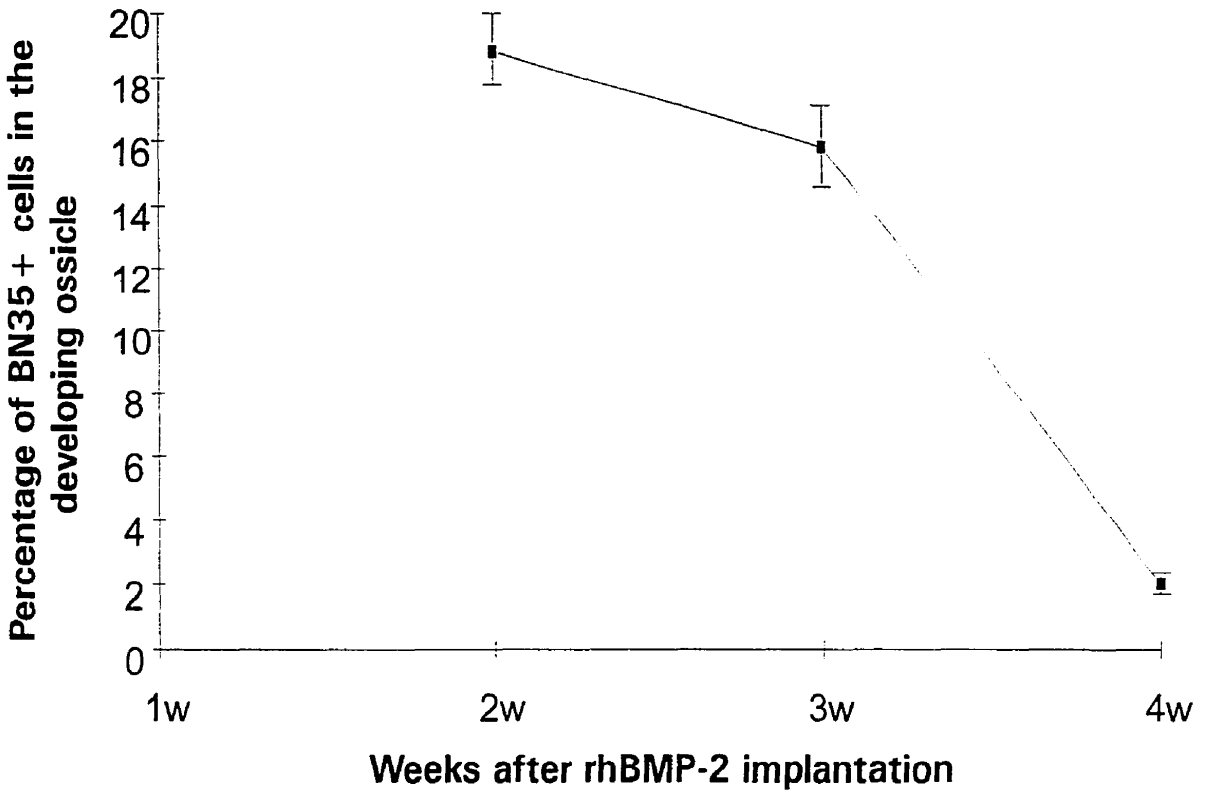
LEGEND TO FIG. 1

Photomicrographs of plastic embedded sections of ossicles 2-8 weeks after implantation of BMP, stained with hematoxylin-eosin (H-E) or Crocott. By 2 weeks, extensive proliferation of fibroblastoid mesenchymal cells was observed between developing foci of cartilage (A-C) (magnification 200x and 100x). The formed bone was extensively remodelled between 3-4 weeks (D) (100x). Typical marrow architecture formed after 5 weeks, and persisted to at least 8 weeks (E). A high frequency of multinucleated giant cells can be seen in preparations obtained from 2-3 week ossicles (F) (Jenner-Giemsa, 250x).


B
E

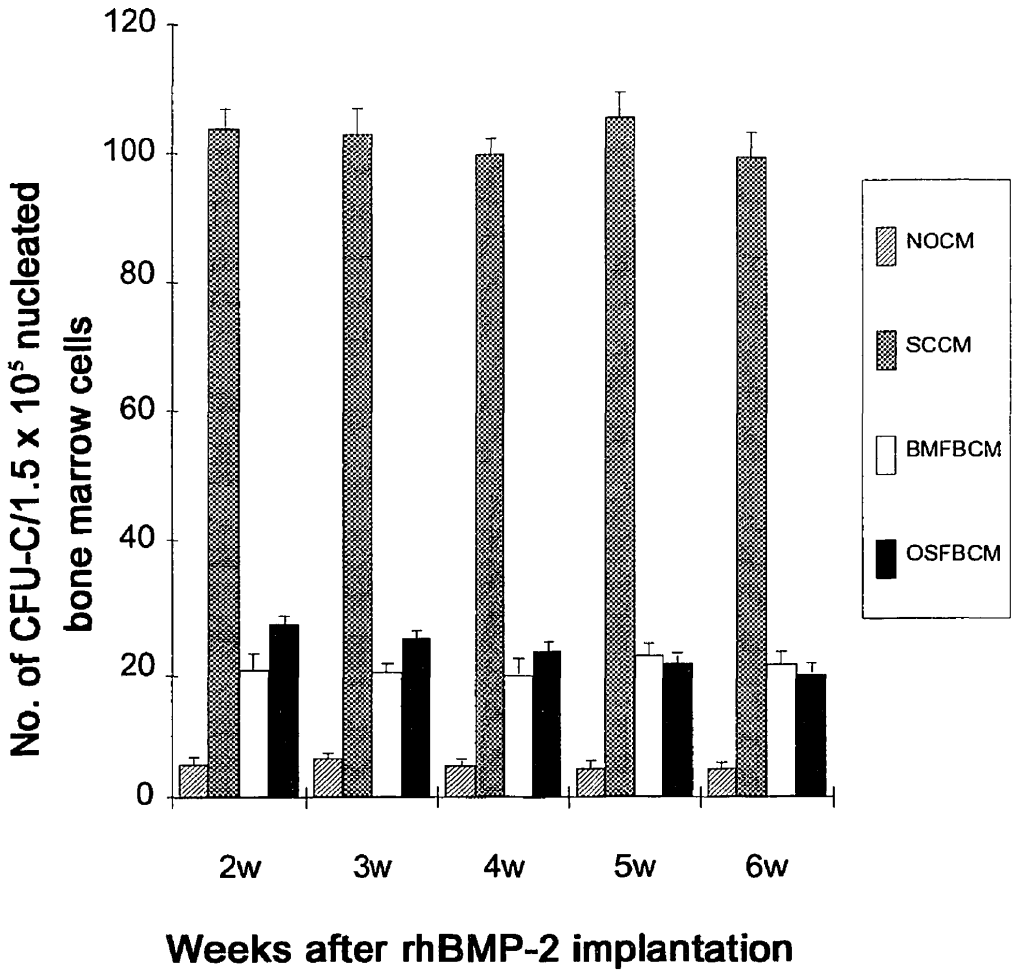
LEGEND TO FIG. 2

The frequency of $BN35^+$ macrophage-lineage cells distributed in the developing ossicle: cytospin preparations, immunostained with anti-BN35 monoclonal antibody. The frequency of $BN35^+$ cells, each data point (mean $\pm$ SD) represents the values obtained from six ossicles from six individual rats.


Percentage of BN35+ cells in the developing ossicle
20
18
16
14
12
10
8
6
4
2
0
1w
2w
3w
4w
Weeks after rhBMP-2 implantation

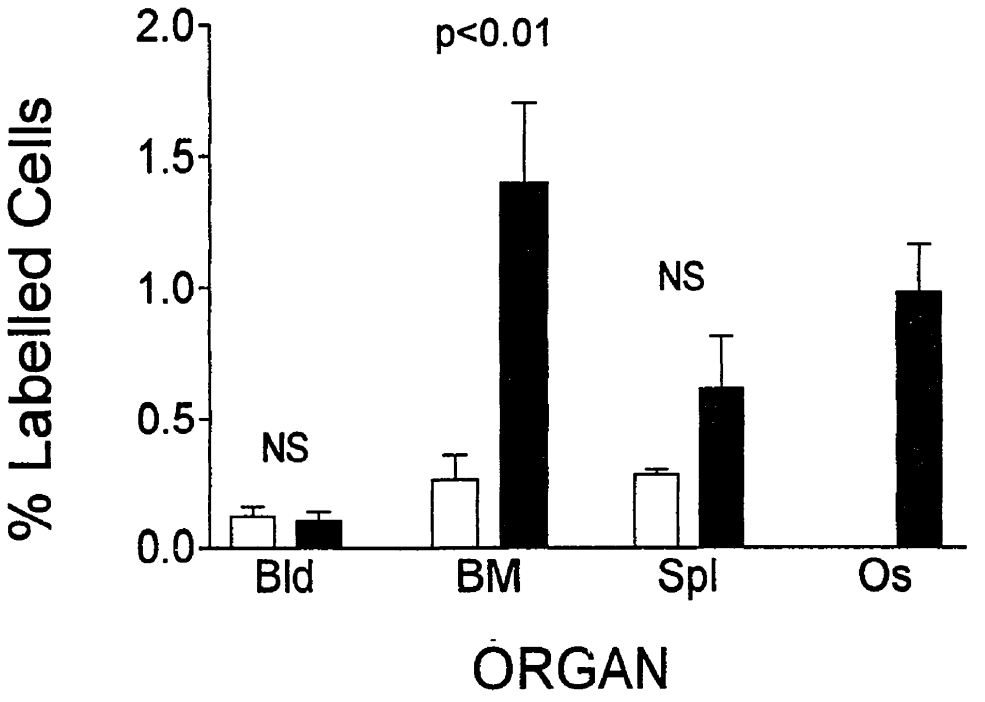
LEGEND TO FIG. 3

The colony stimulating activity in culture supernatants of ossicle-derived fibroblastoid cells. Normal nucleated bone marrow cells ($1x10^5$/ml) were cultured alone in the absence of fibroblastoid cell cultured medium (FBCM) and SCCM (lane 1), with SCCM (lane 2), with ossicle FBCM (lane 3), or with femoral marrow FBCM (lane 4). After 7 days of incubation, colonies were counted by direct inspection with an inverted microscope. Each time point on the graph represents mean $\pm$ SE of results from six individual experiments with preparations of fibroblastoid monolayers from 2-6 week ossicles. The differences between the two conditioned media are not statistically significant at any time (ANOVA, $P > 0.05$).


120
100
80
60
40
20
0
No. of CFU-C/1.5 x 10⁵ nucleated bone marrow cells
NOCM
SCCM
BMFBCM
OSFBCM
2w
3w
4w
5w
6w
Weeks after rhBMP-2 implantation

LEGEND TO FIG. 4

The frequency of CMFDA labelled LacZ BNML cells distributed in different tissues of irradiated rats at 3 days after i.v. injection of 5 x 10^7 cells. Results obtained from flow cytometric (FACScan) analysis of 10^4-10^5 nucleated cells; each data point represents mean $\pm$ SE of 7 individual experiments. BM, bone marrow; SP, spleen; BL, blood; THY, thymus.


% Labelled Cells
2.0
1.5
1.0
0.5
0.0
p<0.01
NS
NS
Bld
BM
Spl
Os
ORGAN

CHAPTER 5

USE OF A cDNA PROBE SPECIFIC FOR THE Y CHROMOSOME TO DETECT MALE-DERIVED CELLS

PREFACE

Hemopoietic chimerism after BMT depends upon long-term survival of stem cells from the donor. Although various methods have been applied to demonstrate this, makers had not been developed for use in a rat model. Based on the published sequence of the Y-chromosome specific murine *sry* gene, we developed a DNA probe to use for such studies (e.g., as in Chapter 4). The characterization and the use of this probe will be discussed herein.

ABSTRACT

A cDNA probe that exhibits specificity for the rat Y chromosome was generated by using a set of primers specific to the murine *Sry* gene, the sex determining region of the Y chromosome. A 459 bp DNA fragment was obtained by PCR amplification from male, but not female, rat genomic DNA. This DNA fragment was purified, cloned using a vector, and digested with EcoRI to yield a 270 bp DNA fragment. This 270 bp cDNA fragment, when used as a probe in Southern blot analysis of rat DNA, was observed to bind to three separate bands of 2.3, 5.0 and 7.0 kb in size. The binding was demonstrated with male, but not female, genomic DNA. Another set of primers was generated to sequences within the 270 bp fragment which produced a PCR product of 104 bp. This DNA fragment, when used as a probe in Southern blot analysis, enabled PCR detection of at least 0.1% male cells in a mixed population of female cells. These cDNA probes should prove useful in studies designed to track cell populations (e.g., tumor metastasis; hemopoietic cells after bone marrow transplantation) in syngeneic male/female pairs. In addition, a cDNA probe that is specific for the rat *Sry* gene might be valuable in studies of fetal male sexual development or the study of spermiogenesis.

INTRODUCTION

The *Sry* gene, located on the short arm of the Y chromosome, encodes for a putative factor that contains a high-mobility-group (HMG) region believed to bend its DNA target and modulate transcription (King and Weiss, 1993). The SRY protein presumably acts on one or more genes downstream to *Sry* to initiate a cascade of events that induces sexual development in the male mammal (Gubbay et al, 1990; Berta et al, 1990; Haqq et al, 1993). Most studies on this process, however, have used either murine (Lovell-Badge, 1993) or human (McElreavey et al, 1993) tissues, and little or no information has been published on the rat, a frequently used animal model in many other areas of research.

Experiments on bone marrow transplantation, immune cell function, and tumor growth often involve tracking donor cells within a recipient. One strategy, which permits the use of animals with identical major histocompatibility antigens and obviates the need to introduce foreign marker genes, is to use male-female combinations. With markers that can detect DNA specific for the Y chromosome, it is possible to trace the progeny of male cells within a female recipient (Agematsu et al, 1990; Gerritsen et al, 1994; Landman-Parker et al, 1993; Lawler et al, 1991; Przepiorka et al, 1991; Santucci et al, 1992; Van Dekken et al, 1989). Although this approach has been used with both mice and human subjects, analogous markers have not been available for the rat, a model commonly used for such studies. Here, we describe the derivation of a DNA probe specific for the rat Y chromosome, and illustrate its use in the detection of male-derived cells by both Southern blotting and the polymerase chain reaction (PCR). This marker ought to prove useful in studies designed to identify donor cells as well, as studies to investigate the role of Sry in male sexual development.

METHODS AND MATERIALS

Animals. Inbred male and female Brown Norway rats of age 6-10 weeks were purchased from Charles River Canada (St. Constant, Québec), and received standard laboratory chow and water ad libitum. Animal care was in accordance with approved institutional guidelines.

Preparation of DNA. Nucleated cells obtained from marrow or thymus of male rats were collected from the interface after Ficoll-Hypaque centrifugation, washed three times with PBS, and 10^7 cells were resuspended in 400 μl TNE buffer (10 mM Tris/pH 7.9; 10 mM EDTA; 10 mM NaCl) plus 400 μl 20% SDS and 40 μl of pronase K (20 mg/ml). The suspension was agitated with a vortex mixer and then incubated at 37°C for 12-15 h. DNA was phenol-extracted, ethanol-precipitated and then dissolved in TE buffer (1 mM Tris, pH 7.9 with 0.1 mM EDTA).

Polymerase chain reaction (PCR). The first PCR product (459 bp) was obtained using primer set #1 with the following sequence, 5' primer: 5' AGATCTTGATTTTTAGTGTTC 3' and 3'primer: 5' TGCAGCTCTACTCCAGTCTTG 3' to regions of the *Sry* gene of the murine "sex determining region" carried on the Y chromosome (Gubbay et al, 1990; Tucker and Lundrigan, 1993; Whitfield et al, 1993). The reaction mixture contained 1 μg genomic DNA, 1 μM of each primer, 200 μM dNTPs, 1X PCR buffer and 2 units Taq polymerase (all from Perkin Elmer) in a final volume of 100 μl. Thirty amplification cycles were performed, each cycle consisting of a denaturation step at 94°C for 1 min, an annealing step at 50°C for 1 min and an extension step at 72°C for 2 min. Following these 30 cycles, an additional extension was performed at 72°C for 7 min. The PCR products were separated by electrophoresis in 0.8% agarose gel and stained with ethidium bromide to assess their size .

The second PCR product (104 bp) was obtained using primer set #2 with the following sequence, 5' primer: 5' CATCGAAGGGTTAAAGTGCCA 3' and 3' primer: 5' ATAGTGTGTAGGTTGTTGTCC 3' to a region toward the 3' end of the 459 bp DNA fragment. These primers were selected using Primer Analysis Software (Oligo Version 4.1 published by National Biosciences, Inc., 3650 Annapolis Lane, Plymouth,

MN 55447). The reaction mixture contained 100-200 ng genomic DNA, 1 μM of each primer, 50 μM of each dNTP, 1X PCR buffer and 1 unit Taq polymerase in a final volume of 50 μl. Optimal conditions for this PCR were determined to be 30 cycles, each consisting of a denaturation step at 94°C for 1 min, an annealing step at 58°C for 1 min and an extension step at 72°C for 2 min. The PCR product was separated by electrophoresis in 2.5% agarose gel and stained with ethidium bromide to assess size.

Cloning and sequencing of the Y chromosome-related PCR product. The 459 bp DNA fragment, which was amplified by male (but not female) rat genomic DNA, was eluted from agarose gel, purified and cloned using the TA cloning™ vector (Invitrogen, San Diego, CA; #K2000-01). Plasmid DNA was extracted following the "miniprep" protocol as described by Sambrook et al (1989). The inserts then were sequenced according to the dideoxy chain termination technique of Sanger et al (1977).

Preparation of the probe for hybridization. The plasmid DNA containing the insert was digested with EcoR1 (5 units/μg), and then electrophoresed in low melting temperature agarose gel. A 270 bp fragment was isolated and labeled by random priming with [α-^{32}P]-dATP according to the procedure of Feinberg and Vogelstein (1983).

Southern blot analysis. Genomic DNA (3-5 μg), previously digested with EcoR1, was electrophoresed on a 0.8% agarose gel and transferred to a Gene Screen Plus™ membrane (Dupont, #NEF-976). After transfer, the membrane was subjected to prehybridization for 4 h at 42°C in a solution containing 50% deionized formamide, 5 X SSPE (1X SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA), 100 μg denatured salmon sperm DNA/ml and 1% sodium dodecyl sulphate (SDS). Hybridization was performed for 12-16 h at 42°C in prehybridization solution containing 10% dextran sulphate, 1X Denhardt's solution, and 1X 10^6 cpm/ml of the [α-^{32}P]-dATP labeled 270 bp DNA probe. As a control for loading, the membrane also was reacted with 1 x 10^5 cpm/ml of [α-^{32}P]-dATP labeled probe that binds to common sequences in the chicken actin gene (Cleveland et al, 1980). The membrane was washed twice in 2X SSC for 20 min at room temperature, once in 0.1X SSC/0.1% SDS for 30 min at 65°C, and exposed to autoradiographic film for 3-5 days.

Detection of male-derived cells by PCR. Male and female cells were mixed in the proportions stated in the "Results", and genomic DNA was prepared. The PCR reaction mixture (100 μl total volume) consisted of genomic DNA (0.5 ng/μl; 50 ng total), 1μM of each primer, 200 μM of each dNTP, 1x PCR buffer containing 2.5 mM $MgCl_2$, and 0.01 u/μl of Taq DNA polymerase (all from Perkin Elmer). Thirty amplification cycles were performed, each cycle consisting of a denaturation step at 94°C for 1.25 min, an annealing step at 58°C for 2.5 min, and an extension step at 72°C for 2.5 min. Following these 30 cycles, the PCR products were separated by electrophoresis in 2.0% agarose gel and stained with ethidium bromide to assess their size. To minimize the occurrence of false-positive signals, the recommendations of Kwok and Higuchi (1989) were followed.

RESULTS

Following the strategies outlined above that used primers derived from the mouse Sry sequence, we first obtained a 459 bp fragment which was amplified from male but not female DNA, as presented in Fig. 1. Determination of the nucleotide sequence of this product enabled comparison with that published for the analogous DNA fragment from murine DNA. Five independent clones were sequenced, and the results are shown in Fig. 2. Overall, a comparison of the two fragments indicated they were 88% homologous, with the greatest degree of overlap in the central region. From bases 1-100 there were 22 differences, but from 101-219 there were only 3; similarly, from 229-350 there were only 5 differences, but from bases 351-451 there were 16 differences.

A 270 bp fragment, obtained by EcoR1 digestion of plasmid DNA containing the 459 bp product, was used as a probe to detect male-derived genomic DNA by Southern blotting at high stringency. As shown in Fig. 3a, the probe reacted with bands of 2.3, 5.0, and 7.0 kb from male but not female DNA. By contrast, the control, a chicken actin probe, bound equally well to both male and female DNA (Fig. 3b). Use of this probe in conjunction with Southern blotting was adequate to detect 5% male-derived thymocytes added to a suspension of female bone marrow cell (Fig. 4), without having reached the lower limit of detection.

Additional primers were generated to the sequences described in Fig. 2 (shown by the open triangles), and were used with PCR to amplify DNA to the level of detection from at least 0.1% male cells in a mixture of female cells (Fig. 5). This reagent also identifies male murine cells, but further species restriction was demonstrated by the lack of a 104 bp band when human-derived male leukocyte DNA was used. Considering the sensitivity of this second set of primers to detect and amplify male-derived DNA, the optimum assay conditions for use of primer set #2 were determined, as shown in Table 1. These conditions included addition of 2.5 mM $MgCl_2$, an annealing temperature of 58°C, and 30-35 cycles in the PCR.

DISCUSSION

This study demonstrates that the 270 bp cDNA probe derived from the rat Y chromosome can be used to detect male DNA in a mixture of female cells. Comparison of the initial rat Y-linked PCR product of 459 bp with that of the mouse Sry gene shows that the two regions are approximately 88% homologous. The lower concentration of 5% for Southern blotting and 0.1% for PCR detection were shown to illustrate the approximate range of activity of the probes, and do not indicate the lower limits that might be attained by further refining the experimental conditions. Subsequently, we have used this tool to confirm engraftment of male bone marrow cells injected into female recipient rats (An et al). In addition, the primers for the 104 bp cDNA have been used to distinguish male and female rat fetuses during mid- to late gestation. These primers also yielded a cDNA product when used against DNA samples from adult male tissues but, produced no product when tested with various female tissues.

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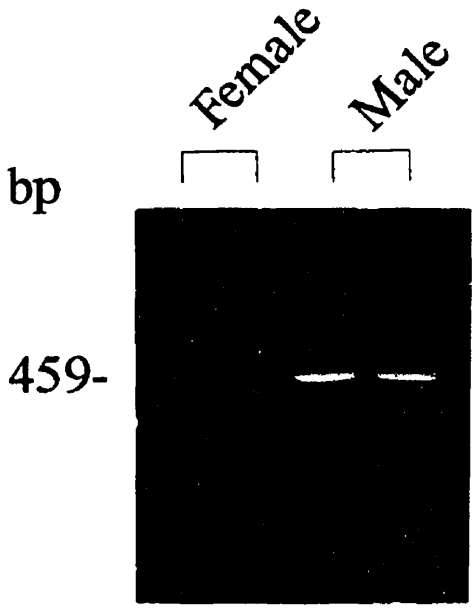
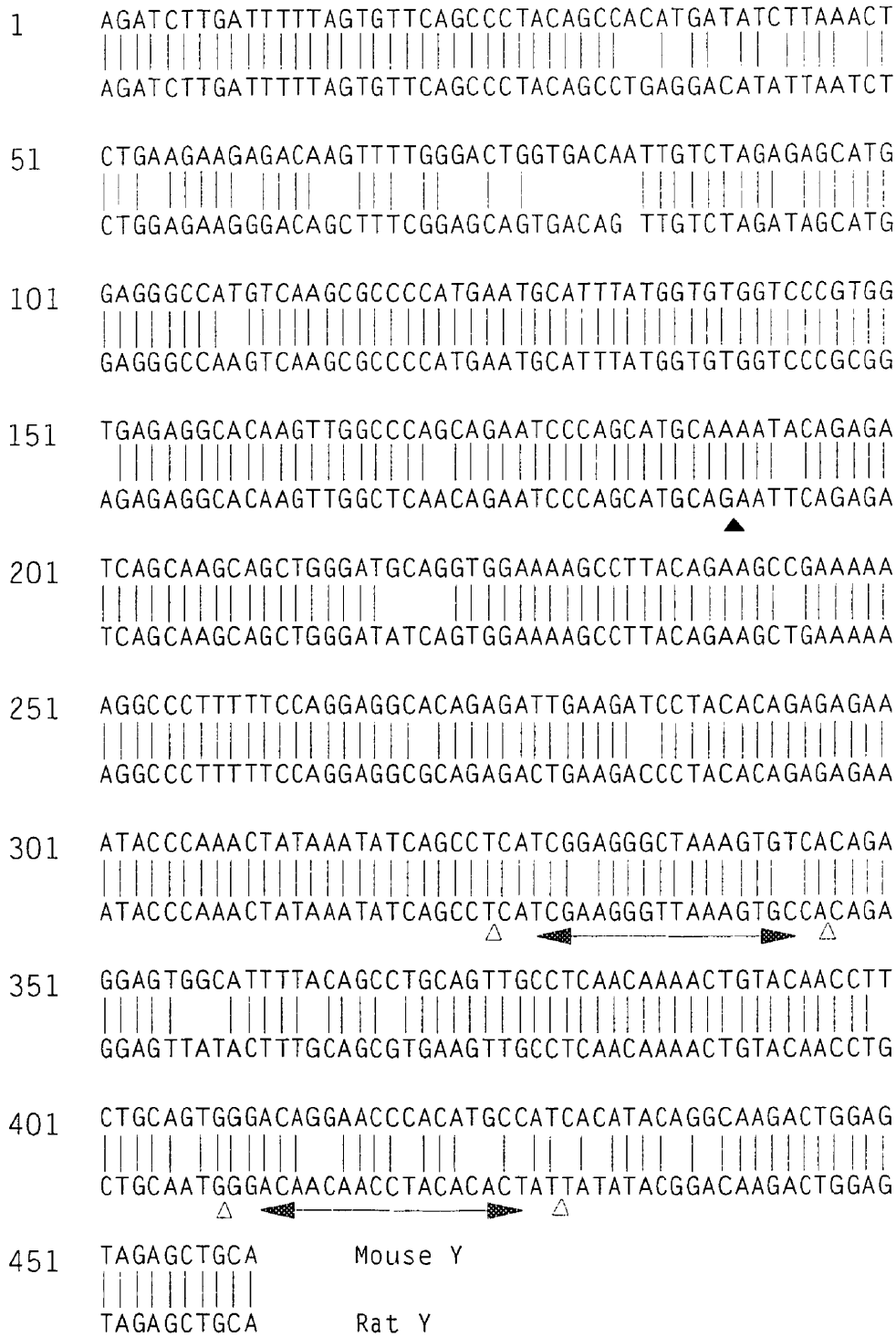
LEGEND TO FIGURE 1

The PCR product obtained from reaction of primer set-one with male and female genomic DNA. Lanes 1-2; female DNA; lanes 3-4; male DNA samples that were obtained as described.


Female
Male
bp
459-

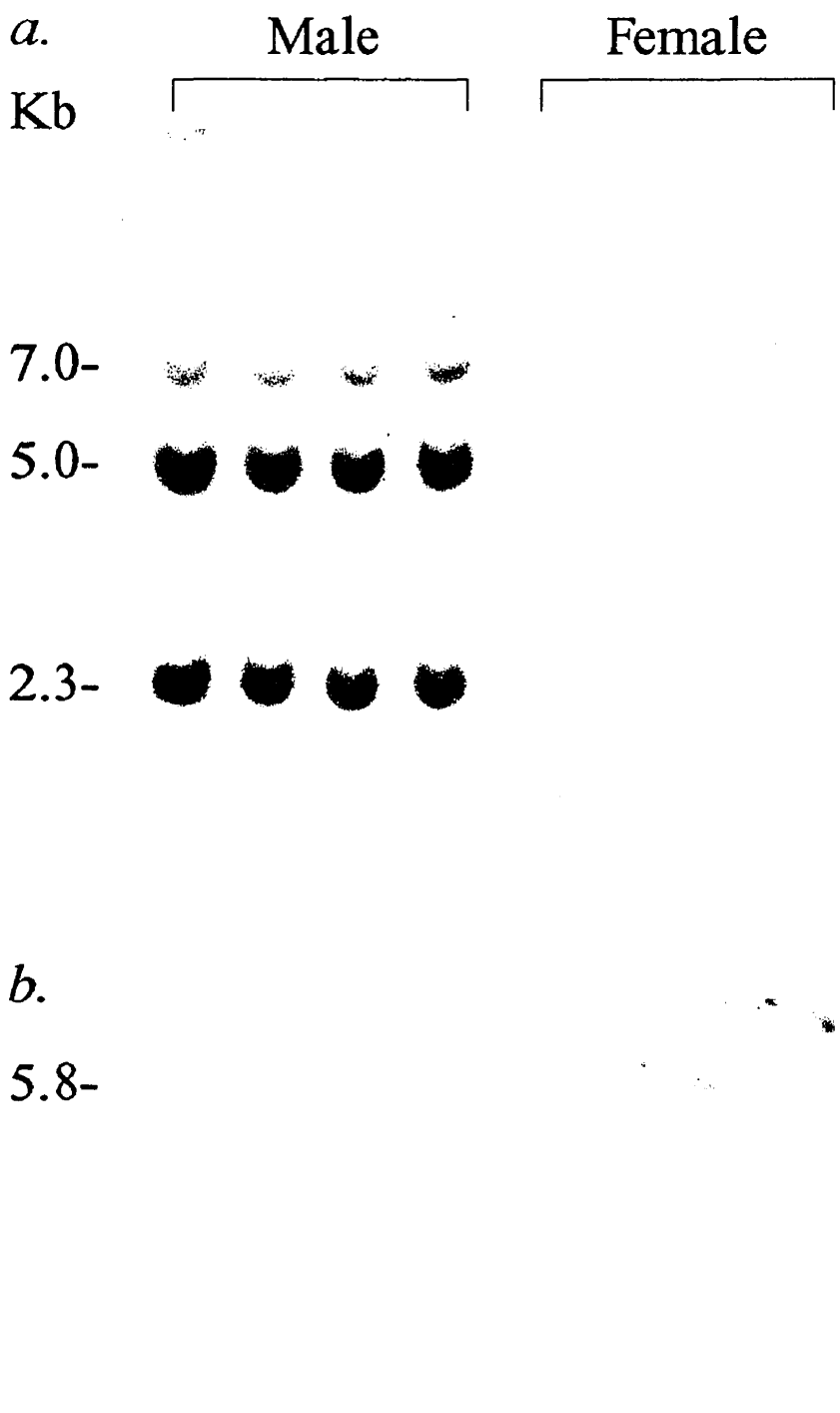
LEGEND TO FIGURE 2

Comparison of the nucleotide sequence of the 459 bp PCR product obtained from rat with that published for the homologous sequence within the murine Sry gene. The solid triangle denotes the EcoR1 cleavage site, 3' to which lies a 270 bp fragment. The open triangles indicate the primers used in the PCR assay to detect to 104 bp sequence.


1
AGATCTTGATTTTTAGTGTTCAGCCCTACAGCCACATGATATCTTAAACT
AGATCTTGATTTTTAGTGTTCAGCCCTACAGCCTGAGGACATATTAATCT
51
CTGAAGAAGAGACAAGTTTTGGGACTGGTGACAATTGTCTAGAGAGCATG
CTGGAGAAGGGACAGCTTTCGGAGCAGTGACAG TTGTCTAGATAGCATG
101
GAGGGCCATGTCAAGCGCCCCATGAATGCATTTATGGTGTGGTCCCGTGG
GAGGGCCAAGTCAAGCGCCCCATGAATGCATTTATGGTGTGGTCCCGCGG
151
TGAGAGGCACAAGTTGGCCCAGCAGAATCCCAGCATGCAAAATACAGAGA
AGAGAGGCACAAGTTGGCTCAACAGAATCCCAGCATGCAGAATTCAGAGA
201
TCAGCAAGCAGCTGGGATGCAGGTGGAAAAGCCTTACAGAAGCCGAAAAA
TCAGCAAGCAGCTGGGATATCAGTGGAAAAGCCTTACAGAAGCTGAAAAA
251
AGGCCCTTTTTCCAGGAGGCACAGAGATTGAAGATCCTACACAGAGAGAA
AGGCCCTTTTTCCAGGAGGCGCAGAGACTGAAGACCCTACACAGAGAGAA
301
ATACCCAAACTATAAATATCAGCCTCATCGGAGGGCTAAAGTGTCACAGA
ATACCCAAACTATAAATATCAGCCTCATCGAAGGGTTAAAGTGCCACAGA
351
GGAGTGGCATTTTACAGCCTGCAGTTGCCTCAACAAAACTGTACAACCTT
GGAGTTATACTTTGCAGCGTGAAGTTGCCTCAACAAAACTGTACAACCTG
401
CTGCAGTGGGACAGGAACCCACATGCCATCACATACAGGCAAGACTGGAG
CTGCAATGGGACAACAACCTACACACTATTATATACGGACAAGACTGGAG
451
TAGAGCTGCA Mouse Y
TAGAGCTGCA Rat Y

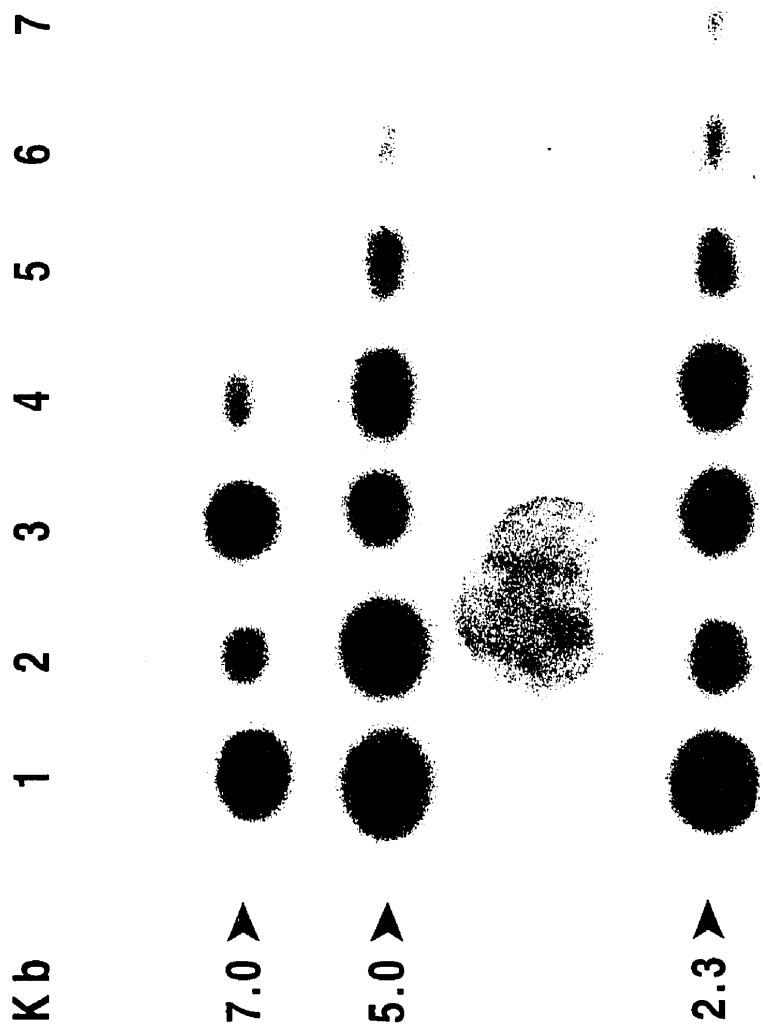
LEGEND TO FIGURE 3

Southern blot analysis of the EcoR1 digested genomic fragments. (a) Blot of genomic DNA prepared from thymi of four male (the four left lanes) and four female (the four right lanes) rats and hybridized with the ^{32}P-labeled 270 bp cDNA probe; (b) Hybridization of the same membrane with a labeled cDNA probe for actin as a control for gel loading.


a.
Male
Female
Kb
7.0-
5.0-
2.3-
b.
5.8-

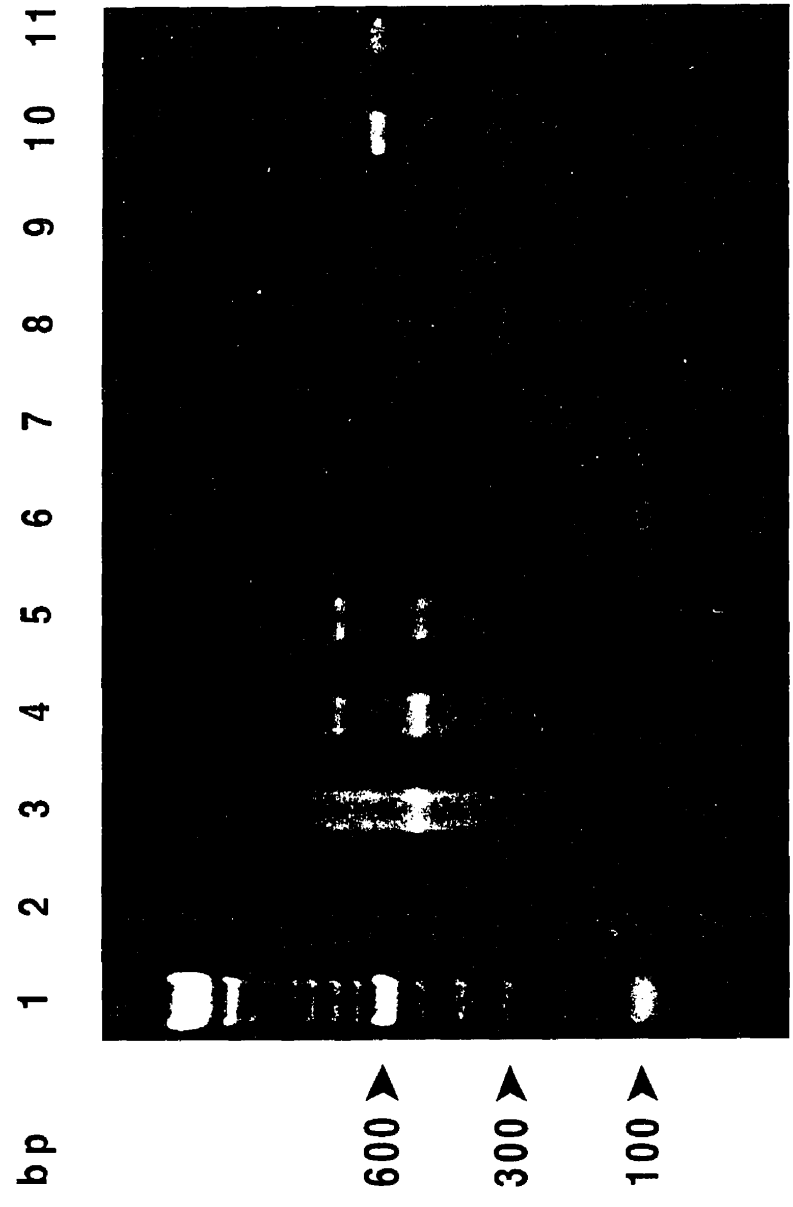
LEGEND TO FIGURE 4

Detection of male thymocytes in a mixture of female bone marrow cells by Southern blotting. A DNA mixture was made with the following ratios of male thymocytes added to female bone marrow cells: lane 1: 100/0; lane 2: 50/50; lane 3: 45/55; lane 4: 40/60; lane 5: 30/70; lane 6: 10/90; lane 7: 5/95.


Kb
1
2
3
4
5
6
7
7.0
5.0
2.3

LEGEND TO FIGURE 5

Use of PCR to detect male thymocyte DNA in a suspension of female bone marrow cells. Male thymocytes were added to female nucleated bone marrow cells and the suspension was prepared for PCR analysis as described in the "Methods". The arrow head denotes the 104 bp position predicted by the primers used in this experiment to detect the male-specific region. Lane 1: standards/100 bp ladder; lane 2: empty; lane 3: 100% female rat DNA; lane 4: 1/1000 male/female rat cells; lane 5: 1/100 male/female rat cells; lane 6: 100% male rat DNA; lane 7: 100% male mouse DNA; lane 8: 100% female mouse DNA; lane 9: blank; lane 10: 100% female human DNA; lane 11: 100% male human DNA.


bp
1
2
3
4
5
6
7
8
9
10
11
600
300
100

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

Many approaches have been used to study the relationship between hemopoiesis and the hemopoietic microenvironment, but most of them have relied wholly upon phenomena observed *in vitro*. The strategy followed in the work of this thesis has been to combine mutually-reinforcing *in vivo* and *in vitro* systems.

1. ORGAN INVASION OF LEUKEMIC CELLS AS AN IN VIVO MODEL TO STUDY INTERACTION WITH THE HEMOPOIETIC MICROENVIRONMENT

Most leukemia cells are originally produced and lodged in the bone marrow. The subline of lacZ BNML cells (LT12NL15) transmit leukemia to rats after i.v. injection, and form colonies *in vitro* in semisolid medium, which presumably are the progeny of the leukemic progenitor cells responsible for maintaining the leukemic population *in vivo* (Yan, 1993). The data presented here demonstrated that (1) the bone marrow is the preferential organ for BNML cells to home and proliferate; the spleen less so, and the thymus not at all; (2) irradiation increases leukemic infiltration into the marrow in the early stage of growth; (3) the frequency of normal hemopoietic precursors was increased in the peripheral blood and spleen, prior to the late stage of terminal leukemia proliferation; (4) the growth of BNML cells was inhibited by the addition of the same spleen cell conditioned medium that stimulates normal myeloid precursors. The molecular mechanisms regulating the uptake and retention of hemopoietic progenitor cells in the marrow and other potentially-hemopoietic organs are not known. Specific adhesion molecules and growth factors (or chemotactic factors) present in the local environment of target organ(s) may play a role (Denkers, 1993, Liesveld, 1994, Verfaillie, 1994). The interactions between tumour cells and endothelial cells of the target organ(s) are very important for determining the initial process of tumour cell homing (Brodt, 1991, Honn, 1992). The expression of a number of membrane glycoconjugates or adhesion molecules such as E-selectin, P-selectin, VCAM-1, and ICAM-1 by endothelial cells were considered important for the binding of metastatic cells (Taichman; 1991; Glinsky,

1993). The activation of endothelial cells usually was induced by inflammation (IL-1, TNF-α, and lymphotoxin, etc.), chemotherapeutic drugs, or irradiation (Shirota, 1991, Cherry, 1994). In our study, we found that the percentage of labelled BNML cells in the irradiated bone marrow 3 days after i.v. injection was 5-6 times higher than in non-irradiated bone marrow. This suggests that damage or activation of endothelial cells by irradiation might enhance the entry of metastatic cells into the bone marrow.

Other stromal elements of the bone marrow play an important role in the lodging and growth of leukemic/metastatic cells that have passed the endothelial barrier. Stromal cells can produce cellular adhesion molecules, humoral factors, and extracellular matrix (Liotta, 1986, Honn, 1992, Gabius, 1994, Cherry, 1994, Verfaillie, 1994). The avid binding of the lacZ BNML cells to bone marrow fibroblastoid cells ($ST3^+/ST4^-$) could contribute to this stage of the process whereby leukemic cells are retained in the bone marrow. A group of cell surface molecules expressed by the stromal cells of hemopoietic organs, in addition to mediating cellular adherent interactions, may also in provide growth/inhibitory signals to cells that were bound (He, 1991, Garnett, 1993). In addition, organ specific ECM may induce tumour cells to express specific homing receptors, and tumour cells in turn may remodel the ECM (Liotta, 1986). The response of tumour cell to these local factors impacts on their survival (van den Hoof, 1988, Lowenberg, 1993).

The shift of CFU-C from the marrow to the spleen in the BNML model might be due to competition between normal and leukemic cells for permissive sites in the bone marrow microenvironment ("niches"); inhibitors of the normal hemopoiesis produced by either BNML cells or abnormal stromal cells under the leukemic condition; activation of stromal cells of the spleen to produce inhibitors of the leukemic cells; or combinations of all of these possibilities.

Future studies using this animal model could lead to new insights into the process of metastatic invasion of the bone marrow by circulating tumour cells. The continued development of MAbs which either recognize organ specific receptors or acceptors on leukemic cells or stromal cells could provide further evidence to understand organ specific homing, which could be applied to develop anti-cell adhesion therapy of cancer metastasis. The work of this thesis could have taken many different directions to

approach the next step, and it was decided to focus on one aspect of the adhesion process that might occur on the extravascular side of the bone marrow.

2. A MARKER FOR MARROW STROMAL FIBROBLASTS MAY BE AN ADHESION MOLECULE RELATED TO THY-1

The nature of stromal-hemopoietic cell associations in the process of hemopoiesis is not well understood. A number of cell surface or ECM adhesion molecules have been found to mediate hemopoietic cell interaction with bone marrow stromal cells. They belong to both the integrin and immunoglobulin (Ig) superfamilies (Williams, 1988). Data presented here suggests that the ST3 antigen is carried on a membrane protein similar to Thy-1, giving biochemical evidence to support previous histological observations. The epitopes probably are not identical, however, based on the following evidence: (1) the distributions of ST3 and Thy-1 in the brain do not wholly overlap (Sharma, 1991); (2) the binding of myeloid precursors/BNML cells to fibroblastoid cells of the bone marrow was partially blocked ($\sim$ 40%) by pretreatment of the stromal monolayer with anti-ST3, but not anti-Thy-1 (TIB103) antibodies; and (3) the two antigens were not extinguished in parallel with prolonged passage of a marrow stromal fibroblastoid cell line.

Evidence has shown that cell-cell adhesion can be mediated by multiple interactions consisting of several different receptor/ligand pairs. An adhesion cascade has been proposed where the engagement of one receptor with its ligand may trigger enhanced adhesion through another (Hynes, 1992, Glinsky, 1993). In the current study, we were unable to completely block the binding of committed myeloid precursors/BNML cells using the anti-ST3 antibody alone, under the described experimental conditions. Among a range of possibilities, this might be due to heterogenous populations of precursors, or other adhesion molecules.

Such adhesive interactions may be necessary for stromal-dependent *in vivo* hemopoiesis, especially since it is an important initial step for cell-cell and cell-ECM recognition (Miyake, 1991). In a normal steady state, adhesion events probably function to maintain primitive progenitor cells quiescent within the marrow, to regulate the

development of hemopoietic cells, and to regulate the release of immature/mature blood cells into the circulation.

The molecular basis of cell adhesion of hemopoietic cells to the bone marrow stroma is complex. A wide variety of adhesion molecules are included in this process, such as $\alpha_4\beta_1$ integrin, CD44, CD55, LFA-1, and the stromal cell-derived ECM molecules such as hemonectin (Campbell, 1987), proteoglycan (Gordon, 1987) or endogenous lectins (Aizawa, 1988). One integrin subtype can bind with at least two alternative ligands, such as $\alpha_4\beta_1$, which interacts with both fibronectin and VCAM-1 (Simmons, 1992). It is not known whether they do so through different binding sites, or the same binding sites but through different ligands, which might include two different molecules possessing a common binding sequence, or one molecule spliced into distinct active forms (membrane form and ECM form); These components (ligand and receptor) could be developmentally regulated, or coordinated with cell renewal, growth, and differentiation (Glinsky, 1993). It is known the extent to which the expression of these adhesion molecules can be modulated by multiple growth factors, cytokines, or lipid mediators, e.g., TGF-β, TNF-α, IL-1, IL-6, and 12(S)-HETE, which are present in the HM or in inflammatory sites (Grossi, 1989, Greenberger, 1991, Simmons, 1992, Glinsky, 1993).

Although the ST3 epitope may function as an adhesion molecule *in vitro,* it may not have any relevance *in vivo*. A possible first step toward building a case for an *in vivo* function for ST3 would be to demonstrate that it is expressed at a crucial period of development when hemopoiesis becomes active in a given organ. This question could be approached in an embryogenesis model. However, hemopoietic function uniquely shifts from yolk sac to liver to spleen to marrow during this period. We chose to exploit the phenomenon of *de novo* marrow formation in the adult under the influence of bone morphogenetic protein. Were the ST3 antigen to be expressed accordingly, as the data presented here suggest, it would argue that it was playing a role in the process.

3. *FUNCTIONAL CHARACTERIZATION OF THE HEMOPOIETIC MICROENVIRONMENT INDUCED BY RECOMBINANT BONE MORPHOGENETIC PROTEIN-2*

By establishing the *de novo* ectopic bone marrow model induced by rhBMP-2, we investigated aspects of how the induced HM contributes to the support of hemopoiesis. The results suggest that (1) the induced ossicles possess a normal marrow architecture; (2) the fully developed HM of the ossicle supports different lineages and stages of hemopoietic cells, including HSCs which have long term (> 6 months) hemopoietic reconstituting activity in sub-lethally irradiated recipients; (3) the microenvironment of the induced ossicle can potentially be colonized by circulating BNML cells; and (4) the stromal fibroblastoid cells derived from the developing ossicle are phenotypically ($ST3^+/ST4^-$) and functionally similar to cells derived from normal skeletal marrow.

The developing hemopoiesis in the BMP-induced ossicle follows a distinct pattern. Early events ($\leq$2 weeks) appear as an inflammatory response, and includes vascular invasion, a high frequency of mononuclear cells (lymphoid cells and macrophages), and fibroblastoid cells, which have high proliferative potential and bear the marrow immunophenotypic marker ($ST3^+/ST4^-$). These early response cells could produce different humoral factors and extracellular matrix components that contribute to a functional HM. The dramatic hemopoietic activity develops in the following 2-3 weeks, and is accompanied by extensive bone remodelling. The lag period of the delayed hemopoiesis coincided with the development of clonogenic myeloid and erythroid precursors detected by *in vitro* assay. The full development of hemopoiesis within the ossicle was confirmed by the existence of three lineages of blood elements, including megakaryocytes, committed precursors (CFU-GM/G/M, CFU-E), and hemopoietic stem cells which had shown long term (> 6 months) hemopoietic reconstituting activity. This was confirmed by Southern blot analysis using the Y chromosome specific probe which we developed to prove that the source of marrow, and possibly spleen cells, in the female recipient of ossicle marrow was of male (ossicle) origin.

Hemopoietic activity in the fully developed ossicle appeared to be identical to that

observed in natural skeletal bone marrow of an adult rat. However, the source of hemopoietic stem cells/progenitors within the ossicle is uncertain. Two possibilities might include circulating stem cells from the natural bone marrow, or stem cells differentiated locally from pluripotent mesenchymal stem cells. Circulating stem cells might be immobilized by humoral factors induced by the inflammatory reaction (local and systemic) to rhBMP-2 implantation. The issue of mesenchymal stem cells is controversial. Experiments have shown that targeted mouse mutations can concomitantly affect both the hemopoietic and the vascular systems. For example, if mice lack TGF-β, a variable defect of blood and vasculature will occur (Dickson, 1995); and in mice null for *flk-1* (a vascular endothelial growth factor receptor), a defect of the organized blood vessels, blood islands and hemopoietic progenitors will occur (Yamaguchi, 1993, Shalaby, 1995). An established chimera-ossicle model, and an analysis of the ossicle marrow cells by using *in situ* hybridization techniques, would provide an opportunity to address this question.

The HM of the fully developed ossicle allows hemopoietic stem cells to egress into the circulation and seed distant sites. This issue was supported by the observation that ossicle-bearing rats recovered from lethal irradiation if the ossicles were protected by a lead shield. The mechanisms of egress of mature or immature hemopoietic cells from the bone marrow are still not clear. This candidate model (intrinsic ossicle marrow transplantation) can be used to study the process and the factors in the regulation of hemopoietic cells released from or homing to the bone marrow.

In its capacity to support hemopoiesis, the induced HM appears to be functionally identical to skeletal bone marrow. By dissecting the stromal elements at different stages of ossicle development, the process of assembly of the complex hemopoietic microenvironment could be reconstructed.

4. PHENOTYPIC CHARACTERIZATION OF STROMAL ELEMENTS OF OSSICLE BONE MARROW.

By *in vitro* studies, fibroblastoid cells, macrophages, endothelial cells, and

adipocytes are considered to be the essential components of the HM (Dexter, 1982), which together support hemopoiesis and regulate cell traffic. Continuing previous studies (Sullivan et al, 1989) from this laboratory, we further examined the fibroblast compartment.

4.1. CFU-F. Dynamically, the potential of stromal cell proliferation can be measured by *in vitro* CFU assays. The frequency of CFU-F was greatest at around 2 weeks of the developing ossicle, and reached a level comparable to femoral bone marrow at 5-6 weeks. The relative reduction of the frequency of CFU-F observed between 3-4 weeks might be due to the dramatic increase in hemopoietic cellularity. The high frequency of CFU-F in the early developing ossicle suggests that they might be important in the formation of the functioning hemopoietic niche. Experiments had shown that cultured bone marrow fibroblastoid cells have the ability to induce bone formation in diffusion chambers (Friedenstein, 1970), as well as the ability to transfer or induce HM formation *in vivo* (Friedenstein, 1974, Tavassoli, 1983). Bone marrow derived CFU-F have been considered, for the most part, not to be transplantable by the intravenous route in mice or humans (Simmons, 1987, Athanasou, 1989, Santucci, 1992), although one report has shown that stromal cells can engraft in properly conditioned hosts (Anklesaria, 1987).

4.2. Immunophenotypic characterization of cells of the ossicle marrow fibroblastoid cells. Comparable characteristics of fibroblastoid cells derived from ossicles and femoral bone marrow tissues include: (1) immunophenotypic similarity ($ST3^+/ST4^-$); (2) significant binding capacity for normal myeloid precursors; and (3) production of colony stimulating activity in the cultured supernatant. Importantly, all of these comparable characteristics occurred as early as 2 weeks after rhBMP-2 implantation, before hemopoiesis was fully developed within the ossicles.

The function of fibroblastoid cells derived from hemopoietic bone marrow may differ from those of other tissues. For example, the colony stimulating activity was produced at a higher level by marrow phenotypic fibroblastoid cells ($ST3^+/ST4^-$) than by

others (e.g., $ST3^-/ST4^+$) (Wang, 1992). The role of the cell surface molecule recognized by the anti-ST3 antibody may have a further role in mediating certain adherent interactions between myeloid committed precursors/BNML cells and marrow fibroblastoid cells. The lineage specific binding capacity of the myeloid (but not the erythroid) precursors to the marrow fibroblastoid cells suggests that different adhesion molecules/growth factors were produced by different stromal cells. The erythroid precursors were developmentally associated with other stromal elements such as macrophages, in the formation of the blood islands. Many of such blood islands were also found in the 5-6 week ossicle marrow. These results provide further evidence that in the marrow, the spatial distribution of hemopoietic cells is determined by the different niches where specific stromal cells lodge. Usually, it is considered that the HM regulates hemopoiesis by its dual functions: it provides adherent sites for hemopoietic cells and provides locally produced, stored, or concentrated hemopoietic regulators.

5. BIOLOGICAL AND CLINICAL IMPLICATIONS

There are few situations where the regeneration of an organ in response to a single recombinant-derived protein can be studied in an adult mammal. These experiments demonstrate that the bone marrow forming as a consequence of the BMP-2 cascade contains the essential hemopoietic and stromal elements. The ectopic hemopoietic ontogeny in this adult model recapitulates events in the embryo. From a more basic perspective, this model provided several opportunities to gain insights into the mechanisms regulating the regeneration of bone/bone marrow in response to developmental regulators (members of TGF-β superfamily) in an adult, and furthermore to study the process and regulation of blood cell production within the induced HM. Resolution at the molecular level of mechanisms regulating the initiation and maintenance of bone/bone marrow tissues could be pertinent to BMT and gene therapy in humans.

5.1. Tissue regeneration and development. The capacity of mammalian BMPs to initiate a programmed cellular cascade that results in the induction of bone and bone marrow may be a functionally conserved process utilized in embryonic development, and recapitulated in post-fetal bone repair. The cell responding to the BMPs are thought to be local mesenchymal elements (Lyons, 1990). In *in vitro* assays these immature mesenchymal cells can differentiate into fibroblasts, reticulocytes, adipocytes or osteoblasts in response to BMPs (Whyte, 1989). Vascular invasion is a prerequisite for bone/bone marrow formation (Reddi, 1994). The major source of the angiogenic factors might be produced by cells (either derived from circulation or local environment) in response to BMP implantation (Folkman, 1987).

Developmentally, the hemopoietic system is derived from ventral mesoderm. Members of the TGF-β superfamily include BMPs have been shown to be mesoderm inducers (Harland, 1994). Evidence had shown that BMP-4 and the dominant-negative type I BMP-2/4 receptor can induce appropriate mesoderm formation (Maeno, 1994, Winnier, 1995). Yet, how cells of the ventral mesoderm differentiate into hemopoietic cells is not yet known. In addition, BMP-4 can induce hemopoietic activity in embryoid bodies formed from mouse embryonic stem cells (Johnasson, 1995). In the BMP-induced ossicle model, other members of the TGF-β family, or unknown molecules, might also be involved. The resolution of the biological reaction in response to rhBMP-2 *in vivo,* will provide more information to understand the developmental regulation in organ/tissue regeneration (Lyons, 1989b, Graham, 1994, Harland, 1994).

5.2. Bone marrow transplantation and gene therapy. BMT is a now being used therapeutically to hasten the healing of major skeletal defects. There is also clinical interest in the use of HSC transplant as a vehicle for gene therapy. Two major limitations of BMT for gene therapy include: (1) acquisition of convenient (quality and quantity) transplantable hemopoietic stem cells, and (2) minimal long-term expression of regulated genes at clinically relevant levels (Brenner, 1995). It is known that the frequency of stem cells is very low in the peripheral blood even after mobilization by different growth factors (Gale, 1992). Gene transfer into stem cells of larger animal models has been less

successful. Although the cord blood stem/progenitor cells could provide greater advantage for gene transfer than that of adult marrow cells, this is still not useful as a source of stem/progenitor cells for autotransplantation in adults, except if the establishment of a cord blood bank becomes generally available.

The ability to generate active bone marrow outside the native medullary space in an adult could be desirable in certain clinical conditions. Although the distribution of subcutaneous fat or other differences between rodents and primates might adversely influence the response of mesenchymal progenitors to BMP-2, autologous transplantation from a *de novo* source might circumvent some of the problems encountered in transplantation of individuals with clinical disease. This approach could be especially useful for individuals who receive chemotherapy or irradiation as the main protocol of treatment. Furthermore, the ability to generate a stable, vascularized multicomponent organoid could add another dimension to strategies for gene therapy of disease of the hemopoietic or non-hemopoietic systems. The HSC of the ossicle may prove to be convenient targets for genetic modification, either to generate progenitors as vehicles to deliver immunomodulatory molecules (e.g., cytokines or growth factors) to human tumours or metastatic cells, or as targets for gene therapy of inherited human diseases.

CHAPTER 7

STATEMENT OF ORIGINALITY

The work described in this thesis has led to the following new knowledge:

1. Using direct methods, we have shown that the rat myeloid leukemia (BNML) grows in the bone marrow earlier than it does in the spleen, and that the normal myeloid precursors are concurrently relocated from the marrow to the spleen.

2. We demonstrated that total body irradiation increased the localization of injected BNML cells to the bone marrow.

3. We have demonstrated that factors released from the heterogeneous population of normal spleen cells, while promoting the *in vitro* growth of normal hemopoietic precursors, inhibit the proliferation of BNML cells. Further work will be required to ascertain if this provides the explanation for the observed *in vivo* events.

4. We have demonstrated for the first time that the ST3 antigen is associated with the Thy-1 molecule. Further work will be required to prove whether it is present on the same protein or is on another closely linked protein of similar molecular weight.

5. We have demonstrated for the first time that the ST3 antigen, either directly or indirectly, contributes to the adhesion reaction between marrow stromal fibroblasts and normal and leukemic myeloid precursors.

6. We have demonstrated for the first time that the ectopic marrow tissue formed at ectopic sites expresses the full range of hemopoietic activity, including stem cells, and also develops stromal fibroblasts in a manner similar to authentic marrow fibroblasts.

7. We have produced the first Y-chromosome specific DNA probe for the rat, which now is in use at Emory University (Atlanta, USA), Erasmus University (Rotterdam, The Netherlands), University of Pittsburgh (Pittsburgh, USA), and other laboratories in Montréal.

CHAPTER 8

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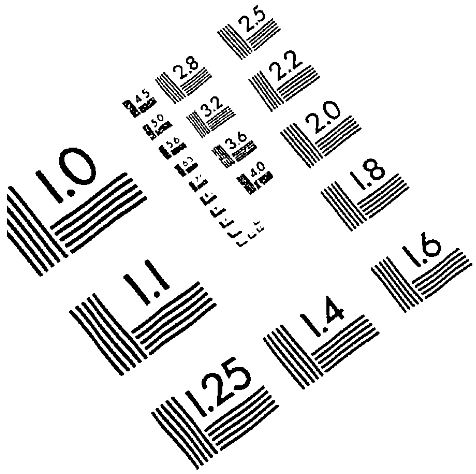
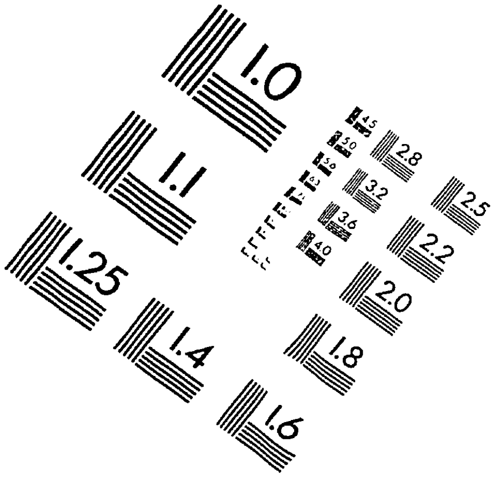
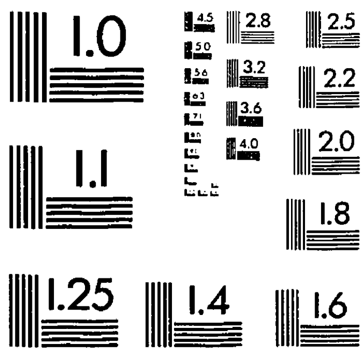
IMAGE EVALUATION TEST TARGET (QA-3)







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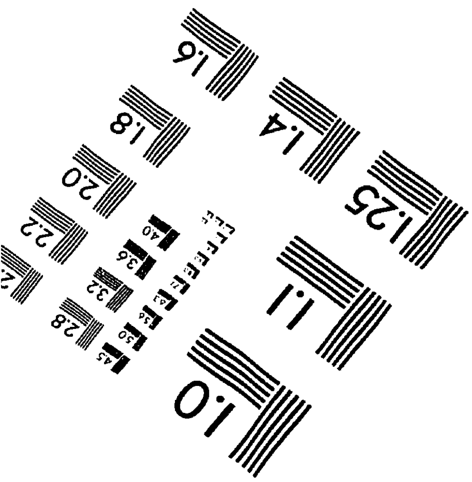

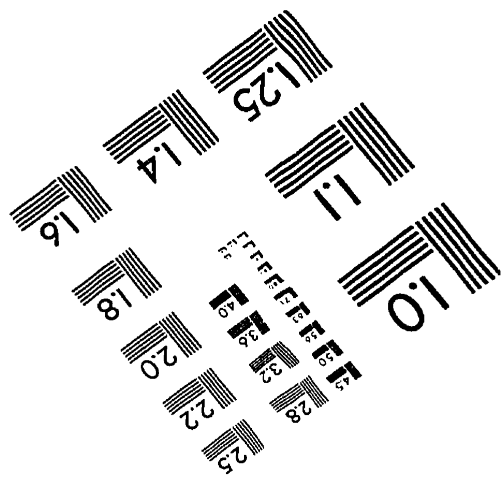

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