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The hematopoiesis-supporting function of bone marrow stroma cells in patients with hematological malignancies

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1 Introduction

1.1 Hematopoiesis

Under physiological conditions, blood cells have a limited life-span and are replaced by a newly produced population every day. In stress situations like bleeding or infection, blood cell production is increased returning to normal when the stress is over. This well-balanced process of blood cell production is termed hematopoiesis. Pluripotent hematopoietic stem cells are a very small subset of blood-forming cells, but they play a pivotal role in the maintenance of the hematopoietic system (Weissman, 2000). Once recruited into the cell cycle, the daughter cells of stem cells may take two different pathways: They may either undergo self-renewal to maintain the stem cell pool, or differentiate into committed progenitor cells which will undergo sequential cell divisions and finally give rise to functionally competent mature end cells. An adequate balance between stem cell self-renewal and differentiation is a prerequisite for the long-term maintenance of hematopoiesis. Recent observations highlight the importance of the hematopoietic microenvironment in this process (Dorshkind, 1990; Lichtman, 1981; Mayani et al., 1992b)**.**

1.2 The hematopoietic microenvironment and its in vitro model - the long-term bone marrow culture system

While blood cells circulate freely throughout the body, proliferation and differentiation of hematopoietic stem cells are restricted to the hematopoietic tissues. In lethally-irradiated animals reconstituted with donor marrow cells, hematopoietic colonies exclusively develop within the bone marrow and the spleen (Till and McCulloch, 1961). Mice of strain Sl/Sl^{d} which have a congenital hypoplastic anemia as the result of a deficient hematopoietic microenvironment, can be cured by transfusion of allogeneic normal spleen tissue but not by normal hematopoietic stem cells (Bernstein, 1970). These facts suggest the existence of a regulatory hematopoietic microenvironment.

In the early 1970's, a bone marrow culture model was developed allowing the continued production of blood cells in vitro (Dexter et al., 1977). This long-term bone marrow culture system provided direct evidence for the regulatory function of the stromal microenvironment in the hematopoietic process. Long-term bone marrow cultures are liquid cultures containing

culture medium, horse serum, fetal bovine serum and bone marrow cells which, in the course of several weeks, form an adherent layer of predominantly non-hematopoietic cells on the bottom of the culture vessels. Hematopoietic stem cells burrow in the adherent layer, proliferate and, on phase-contrast microscopy, become visible as clusters of dark cells termed 'cobble stone areas'. Under the regulatory influence of the adherent layer the stem cells give rise to maturing hematopoietic cells which are being produced for a period of 8-12 weeks, followed by a gradual decline in cell production. After its initial description, several modifications have been made to adapt this culture system to the needs of specific scientific questions. Greenberger added hydrocortisone to the cultures in order to facilitate the development of a functional adherent layer which, in the absence of this hormone, is largely dependent on the batch of horse serum chosen (Greenberger, 1978). In some mouse strains, myeloid cell production can thus be maintained for more than a year (Greenberger and Moloney, 1978). Growth of erythroid cells may be obtained by supplementing the cultures with anemic mouse serum (Dexter et al., 1981). If hydrocortisone and horse serum are omitted and β-mercaptoethanol is added to the cultures, B-lineage lymphoid hematopoiesis can be maintained and studied for prolonged periods of time (Whitlock and Witte, 1982).

The long-term bone marrow culture system is considered to be the best in vitro model for hematopoiesis since it allows the production of blood cells in the absence of exogenously added growth factors. It has been reported that the cultures produce the whole range of differentiated progeny although further maturation is limited to the granulocyte lineage (Dexter, 1979). Eaves et al. reported that the most primitive hematopoietic stem cells are located within the stroma layer (Eaves et al., 1991). The primitive hematopoietic stem cells are maintained in a quiescent state if not perturbed, but they enter the cell cycle when the culture medium is changed (Cashman et al., 1985; Cashman et al., 1990). Whilst the nonadherent cell population in the supernatant is made up of terminally differentiated granulocytes and macrophages, clonogenic progenitor cells (particularly those differentiating along the granulocyte-macrophage lineage) can be detected both in the nonadherent and the adherent layer with continuous cycling irrespective of the timing of the medium change (Cashman et al., 1985).

These observations imply the existence of a complex system of positive and negative regulatory factors orchestrating the distribution, differentiation, maturation and cycling behavior of different hematopoietic cell populations. Conclusive evidence has been presented

that these regulatory influences emanate from the adherent layer which constitutes the hematopoietic microenvironment in the long-term bone marrow cultures. The hematopoietic microenvironment is composed of hematopoietic and non-hematopoietic cells which secrete cytokines, elaborate adhesion molecules and produce the extracellular bone marrow matrix (Dorshkind, 1990; Lichtman, 1981; Mayani et al., 1992b; Verfaillie et al., 1994) .

Similar to the bone marrow stroma in vivo, the adherent layer of a long-term bone marrow culture consists of four main cell types. The major components are fibroblasts and macrophages. Fibroblasts comprise 45 - 75% of the total cell number (Zhang et al., 1999) and may be subdivided into two distinct morphological subtypes, one having bipolar, spindle-cell morphology and the other a polygonal appearance. Both types have the ability to produce collagens I, II, III, IV, VI, VII, fibronectin, laminin, CD44, vascular cell adhesion molecule-1 (VCAM–1), CD68, and a variety of cytokines. Macrophages are also present in significant numbers, comprising 25-35% of the adherent cells. Two morphological variants are recognized, one with round shape and abundant intracytoplasmic debris as evidence of phagocytosis, and the other with elongated and flattened shape lacking phagocytosed debris. Antigens expressed by stromal macrophages include CD14, CD35, CD44, CD68, VCAM-1, intercellular cell adhesion molecule-3 (ICAM-3), vimentin and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Wilkins and Jones, 1995). Endothelial cells comprise 5 - 20% of the cells of the adherent stroma layer. They contribute to the production of cytokines such as granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), GM-CSF, kit-ligand, interleukin-6 (IL-6) and leukemia inhibitory factor (LIF), and bind hematopoietic cells through integrins and selectins (Rafii et al., 1997). Adipocytes constitute a minor proportion of stroma cells. They are derived from adventitial reticular cells and can be induced by hydrocortisone (Gartner and Kaplan, 1980). Within these cell populations, fibroblasts, endothelial cells and adipocytes are of non-hematopoietic origin, while macrophages are derived from the blood-forming system (Agematsu and Nakahori, 1991; Castro-Malaspina et al., 1980; Laver et al., 1987; Perkins and Fleischman, 1988).

Although direct cell-to-cell contact has been shown to exist between stromal and hematopoietic cells by electron microscopy (Weiss, 1976) as well as electrophysiological and dye-transfer techniques (Dürig et al., 2000), other mechanisms are also important in the interaction between hematopoietic cells with the microenvironment including signalling via

soluble or cell-associated cytokines or interactions with the extracellular matrix produced by stroma cells.

Early investigations failed to detect cytokines in significant amounts in unconcentrated stroma conditioned medium (Dexter et al., 1990; Williams et al., 1978). More recent data, however, point to the presence of both stimulatory and inhibitory hematopoietic growth factors in the long-term culture system (Deschaseaux et al., 1994; Eaves and Eaves, 1988; Gibson et al., 1995; Gulati and Nath, 1994). Possible reasons for this controversy include 1) rapid utilization of growth factors in the cultures, 2) growth factors acting synergistically at low concentrations, and 3) binding of cytokines to stroma cell membranes or extracellular matrix proteins (Quesenberry et al., 1991). Further complexity is generated by the fact that 1) microenvironmental cells do not only produce hematopoietic cytokines but are also regulated by them (Table 1), 2) cytokines interact with each in such a way as to induce functional alterations, e.g. by interaction with other regulators stimulatory factors may turn into inhibitory ones or vice versa (Caux et al., 1990), and 3) some cytokines can regulate the proliferation and synthesis of the extracellular matrix components (Strobel et al., 1997), or conversely, some extracellular matrix components can induce the production of growth factors (Gordon, 1994). The complexity of the interactions between cells, cytokines and the extracellular matrix makes it difficult to clarify the exact mechanisms by which microenvironmental cells regulate hematopoietc cells.

Table 1. Growth factors acting on or produced by bone marrow stroma cells

Stroma cell type	*Factors acting on stroma cells*	*Factors produced by stroma cells*
Fibroblasts	IL-1, IL-6, IL-7, G-CSF, IFN-γ, PDGF, TGF-β, TNF-α	IL-1, IL-6, IL-7, IL-8, G-CSF, M-CSF, GM-CSF, SCF, IFN-β
Macrophages	IL-1, IL-4, IL-8, TGF-β, TNF-α, M-CSF	IL-1, IL-6, IFN-α, G-CSF, M-CSF, GM-CSF, PDGF, TGF-β, TNF-α

Abbreviations: IL: interleukin; G-CSF: granulocyte colony-stimulating factor; M-CSF: macrophage CSF; GM-CSF: granulocyte-macrophage CSF; IFN: interferon; PDGF: platelet-derived growth factor; TGF: transforming growth factor; TNF: tumor necrosis factor; SCF: stem cell factor.

Hematopoietic cells and non-hematopoietic stroma cells can produce a complex array of extracellular matrix molecules including collagens, proteoglycans and glycoproteins (Verfaillie et al., 1994). Collagens include types I, III, IV, and VI. They are thought to be produced by non-hematopoietic stroma cells. Other matrix molecules include fibronectin, laminin, thrombospondin and hemonectin (Giles et al., 2002). Proteoglycans are heterogeneous macromolecules consisting of a core protein and multiply sulphated side chains of repetitive disaccharide units, referred to as glycosaminoglycans. Forty percent of the

glycosaminoglycans are hyaluronic acid, others are composed of heparan sulfate, chondroitin sulfate or dermatan sulfate. Glycoproteins may act as cell adhesion molecules. They are expressed by both hematopoietic and non-hematopoietic cells and fall into six structurally distinct superfamilies including integrins, selectins, sialomucins, immunoglobulins, CD44 and cadherins.

The mechanisms by which extracellular matrix molecules participate in the regulation of hematopoiesis are complex. First, they provide an anchor site for hematopoietic cells. Second, they function locally as stimulatory or inhibitory factors (Nilsson et al., 2003; Siczkowski et al., 1992; Weinstein et al., 1989; Wight et al., 1986). Third, they may be shed into the culture medium where they may act as soluble regulatory factors (Gupta et al., 1996). Fourth, they may indirectly regulate hematopoiesis by binding cytokines secreted by stroma cells (Gordon et al., 1987; Roberts et al., 1988).

The hematopoietic microenvironment is of pivotal importance for the function of hematopoietic stem cells. Schofield developed the concept of the 'stem cell niche' (Schofield, 1978) which assumes that the capacity of the progeny of pluripotent hematopoietic stem cells to retain their pluripotentiality is not an intrinsic property of the stem cells proper, but the result of an interaction with specific sites within the hematopoietic microenvironment in which the stem cells lodge. A daughter cell, if placed in a stem cell niche, would remain pluripotent. If a daughter cell were to migrate from the niche, it would acquire an increasing probability of differentiation. The concept of specific niches in the microenvironment has subsequently been refined and extended by other investigators (Verfaillie, 1998). Selective adhesion of cells of a certain developmental stage to extracellular matrix components or stroma cells will result to exposure to specific conditions ('milieu') which will be conducive to the development of the cells. Thus, the orderly progression of hematopoiesis may be guaranteed by the transition of cells from one type of niche to another. Evidence has been presented that, in vivo, stem cells preferentially lodge close to the osteoblast-rich endosteal surface (Calvi et al., 2003; Zhang et al., 2003). As differentiation and maturation proceed, the cells are migrating closer to the center of the marrow cavity where they enter the blood stream.

In summary, hematopoiesis is a complex process in which pluripotent stem cells proliferate and differentiate to generate the different compartments of mature blood cells. Four main types of stroma cells, more than 30 hematopoietic cytokines, more than 20 different adhesion molecules and a complex extracellular matrix constitute an interactive network, the so-called

hematopoietic microenvironment, which is the anatomical and functional basis for long-term balanced hematopoiesis.

1.3 Functional analysis of the hematopoietic microenvironment in vitro

Depletion of long-term bone marrow cultures of hematopoietic cells leads to the generation of pure stroma layers whose overall hematopoiesis-supporting function may then be analysed using standardized indicator cells. In these so-called 'two-stage long-term bone marrow cultures', stroma layers are grown from bone marrow samples of interest, irradiated to eliminate the endogenous hematopoiesis, and recharged with a standard inoculum of indicator cells (usually bone marrow mononuclear cells or purified progenitor cells). This provides the possibility to study the effect of the stroma on the maintenance of very primitive progenitor cells without the confounding effects of an ill-defined mixture of hematopoietic cells. The most commonly used endpoint is to measure the number of colony-forming hematopoietic cells which are generated after a 5-week culture period on the irradiated stroma layer. Although not identical, this endpoint yields similar results to those obtained using more cumbersome assay systems such as limiting dilution analysis or the enumeration of cobble stone area forming cells (Sutherland et al., 1989). All three endpoints are commonly used as a surrogate marker for hematopoietic stem cells. When the number of indicator cells added to the adherent layer is kept constant, the number of colony-forming cells in the cultures after 5 weeks is a quantitative measure for the hematopoiesis-supporting capacity of the stroma.

1.4 The hematopoietic microenvironment in hematological malignancies

Hematological malignancies are clonal disorders resulting from neoplastic transformation of hematopoietic stem or progenitor cells. Similar to their normal counterparts, proliferation and differentiation of the transformed cells still underlie modulation by the hematopoietic microenvironment. The transformed cells and their progeny infiltrate the hematopoietic microenvironment and their products such as cytokines, extracellular matrix components, adhesion molecules or material transferred by direct cell-cell contact may influence the function and composition of the stromal microenvironment. Schematically, the microenvironment may be affected in three different ways in hematological malignancies (Dührsen and Hossfeld, 1996):

1) Neoplastic cells induce reversible changes in stroma function or composition which may favor the growth of malignant cells ('malignancy-induced microenvironment').
2) Elements of the malignant clone become part of the stroma. Sometimes they preferentially promote the growth of neoplastic cells and inhibit the proliferation of normal cells ('malignant microenvironment').
3) A primary stromal abnormality causes an inability to control regular blood cell formation which favors the emergence of 'opportunistic' neoplastic cell clones ('malignancy-inducing microenvironment').

1.5 Goals of the present investigation

The goal of the present investigation was to describe and compare alterations in stroma function in distinct hematological malignancies, and by use of cell fractionation techniques, identify the types of stroma cell responsible for these alterations. To this end we chose three hematological neoplasms all of which were characterized by bone marrow infiltration, but which differed in a number of other parameters.

Acute myeloid leukemia (AML) is a stem or progenitor cell disease with replacement of normal hematopoiesis by leukemic blasts. The disease is heterogeneous with respect to cytogenetic and immunophenotypic characteristics. According to the 'French-American-British' (FAB) classification it is subdivided into seven morphological subtypes. Abnormalities involve both the myeloid, erythroid and megakaryocytic lineages for which the long-term bone marrow culture condition provides very good growth conditions, thus making it a good model for hematopoietic changes occurring in AML in vivo.

Despite heterogeneous genetic, morphological, biological and clinical features, all forms of myelodysplastic syndromes (MDS) are considered to be hematopoietic stem cell disorders accompanied by ineffective hematopoiesis and multi-lineage cytopenia. According to the FAB classification five different types of MDS may be distinguished. This group of diseases constitutes a preleukemic disorder in which the neoplastic clone may or may not progress to full-blown acute leukemia. Due to a large number of common features between AML and MDS, many hematologists consider them as parts of a continuous disease spectrum rather than distinct disorders.

Multiple myeloma (MM) is a post-germinal center B-cell tumor with a clonal proliferation of transformed plasma cells in the bone marrow. Osteolytic bone lesions, anemia, immunodeficiency and renal impairment constitute the main features of this disease. Being a lymphoid neoplasm multiple myeloma is clearly distinct from AML and MDS.

2 Patients, materials and methods

2.1 Patients

Bone marrow samples were obtained with informed consent from patients undergoing diagnostic bone marrow biopsy. In general, the number of cells harvested was sufficient to prepare one set of cultures as described in section 2.4 and Figure 1. Parallel cultures proving reproducibility of the experimental procedure were limited to patients with particularly ample bone marrow aspirates (cf. 3.1.2.4).

2.1.1 Control subjects

The control subjects underwent bone marrow biopsy for untreated lymphoma or other diseases. Morphology and surface marker expression using a fluorescence-activated cell analyser were employed to exclude bone marrow involvement. The clinical details of 11 patients used as controls in section 3.1 and 17 patients used as controls in section 3.2 are listed in Tables 2 and 3, respectively. The age of this population ranged from 20 to 84 years (median 59), and there were 15 males and 13 females.

2.1.2 Patients with acute myeloid leukemia

Unless otherwise stated, all cases of AML were newly diagnosed fulfilling the diagnostic criteria of the FAB classification (Bennett et al., 1976). The clinical details of 6 patients analysed in section 3.1 and 15 patients analysed in section 3.2 are listed in Tables 4 and 5, respectively. The age of this population ranged from 33 to 80 years (median 65), and there were 7 males and 8 females.

2.1.3 Patients with myelodysplastic syndromes

The diagnosis of MDS was based on the criteria of the FAB cooperative group (Bennett et al., 1982). Three of the patients were newly diagnosed, three had long-standing disease, and none had received chemotherapy. In this group, the age range was 60 to 77 years (median 67), and there were 4 males and 2 females (Table 6).

2.1.4 Patients with multiple myeloma

All multiple myeloma patients were untreated and in stage III according to the criteria of the Southwest Oncology Group (Durie and Salmon, 1975). The clinical details are given in Table 7. The age ranged from 47 to 67 years (median 59), and there were 5 males and 4 females.

Table 2. Clinical and hematological data of control patients studied in section 3.1

Patients					*Peripheral blood counts*		
No.	*Initials*	*Age (years)*	*Gender*	*Disease*	*WBC x 10^9/L*	*Hb g/dL*	*Platelets x 10^9/L*
1	BE	66	F	DLBCL	5.5	12.9	313
2	SKH	58	M	MCL	7.5	9.4	133
3	PT	55	M	DLBCL	4.7	10.8	302
4	BS	32	M	DLBCL	5.6	14.7	293
5	GO	82	M	DLBCL	8.2	14.8	154
6	HU	56	M	HD	11.6	10.8	297
7	AM	20	F	HD	7.0	13.7	429
8	RD	55	F	Osteomyelitis	6.9	14.2	317
9	RG	67	F	Polyarthritis	4.6	11.3	80
10	BU	32	M	Splenomegaly	6.7	14.5	244
11	SG	64	F	B-NHL	11.6	12.7	252

Table 3. Clinical and hematological data of control patients studied in section 3.2

Patients					*Peripheral blood counts*		
No.	*Initials*	*Age (years)*	*Gender*	*Disease*	*WBC x 10^9/L*	*Hb g/dL*	*Platelets x 10^9/L*
1	SM	70	M	DLBCL	6.3	14.3	225
2	MH	50	M	Cutaneous FL	9.3	14.9	194
3	WKD	69	M	T-NHL	5.5	14.0	249
4	BM	85	F	MZL	6.8	13.9	270
5	KA	76	F	MZL	9.2	14.0	231
6	OM	53	M	FL	13.4	12.5	309
7	BE	64	F	Iron Deficiency	4.9	10.2	181
8	SK	24	F	HD	13.7	12.5	434
9	RE	84	M	Cutaneous T-NHL	7.4	13.3	214
10	PH	62	F	B-NHL	15.7	13.7	227
11	BJ	62	M	PCNSL	8.6	14.5	185
12	BM	39	M	DLBCL	9.1	13.6	356
13	RI	38	F	HD	6.4	11.3	285
14	WK	59	F	Burkitt's NHL	5.9	11.6	78
15	VL	37	M	HD	6.5	15.3	275
16	HE	36	M	Leukopenia	3.6	16.0	197
17	DB	59	F	Leukopenia	3.1	11.7	184

Abbreviations in Tables 2 and 3: DLBCL: diffuse large B cell lymphoma; F: female; FL: follicular lymphoma; Hb: hemoglobin; HD: Hodgkin's disease; M: male; MCL: mantle cell lymphoma; MZL: marginal zone lymphoma; NHL: non-Hodgkin's lymphoma; PCNSL: primary central nervous system lymphoma; WBC: white blood cells.

Table 4. Clinical and hematological data of newly diagnosed AML patients studied in section 3.1

Patients				*Disease characteristics*		*Peripheral blood counts*		
No.	*Initials*	*Age (years)*	*Gender*	*FAB type*	*Marrow cytogenetics*	*WBC x 10^9/L*	*Hb g/dL*	*Platelets x 10^9/L*
1	HB	58	F	M2	t(8;21)(q22;q22)	10.8	10.1	69
2	PA	53	F	M1	Normal	17.8	9.9	216
3	FRJ	32	M	M4	t(10;14)(p13;q22)	21.1	11.1	65
4	PSBG	69	F	M4	+3, +8, del(21)	25.6	11.8	95
5	WW	50	M	M0	t(4;12)(q11;p13)	96.3	10.4	269
6	DP	41	F	M4	+8, t(9;11)(p21-22;q23)	30	9.2	31

Table 5. Clinical and hematological data of newly diagnosed AML patients studied in section 3.2

Patients				*Disease characteristics*		*Peripheral blood counts*		
No.	*Initials*	*Age (years)*	*Gender*	*FAB type*	*Marrow cytogenetics*	*WBC x 10^9/L*	*Hb g/dL*	*Platelets x 10^9/L*
1	WL	50	F	M4	Normal	93	9.2	76
2	TR	68	F	M4	t(1;5)(p32;q35)	96	9.8	60
3	DK	80	F	M4	inv(16)(p13q22)	20.5	13.0	53
4	DGV	71	F	M5	Normal	47.5	10.5	101
5	RA	65	M	M1	t(1;8)(p36;q24.1), t(5;15), add(19)(q13)	3.7	9.4	60
6	ME	53	M	M2	add(11)	17.1	7.3	106
7	GR	63	M	M4	+X, +Y, -6, -21, add(21)(q13)	2	8.2	20
8	FM	70	F	M4	-X, +8, t(8;21)(q22;q22)	8.4	7.2	34
9	IM	72	M	M4	+8	36.2	9.7	36
10	GA	80	F	M4	Normal	14.4	7.8	29
11	ZD	66	F	M4	Normal	1.5	9.1	162
12	FR	42	M	M1	-Y, -2, del(3)(p12), del(7)(q21q31), -11, -12, +3mar	9.2	5.5	7.5
13	BH	37	F	M2	Normal	21.1	8.5	71
14	RJ	45	M	M4	Normal	2.5	11.6	328
15	BV	33	M	M0	Normal	31	8.0	47

Abbreviations in Tables 4 and 5: F: female; FAB: French-American-British classification; Hb: hemoglobin; M: male; WBC: white blood cells.

Table 6. Clinical and hematological data of MDS patients studied

Patients				*Disease characteristics*		*Peripheral blood counts*		
No.	*Initials*	*Age (years)*	*Gender*	*FAB type*	*Marrow cytogenetics*	*WBC x 10^9/L*	*Hb g/dL*	*Platelets x 10^9/L*
1	CG	77	M	RA	-Y	2.0	10.8	88
2	TG	65	F	RARS	Normal	4.8	11.4	194
3	PM	70	M	RA	del(13q)(q12q14)	9.9	11.1	141
4	RW	60	F	RARS	Normal	6.6	11.0	386
5	KC	67	M	RA	-Y, +8	5.1	6.7	58
6	PR	67	M	RAEB	del(3)(p11p21), -4, -5, -9, +mar	3.7	8.1	4

Table 7. Clinical and hematological data of newly diagnosed multiple myeloma patients studied

Patients				*Disease characteristics*			*Peripheral blood counts*		
No.	*Initials*	*Age (years)*	*Gender*	*Monoclonal immunoglobulin*	*Renal disease*	*Osteolyses*	*WBC x 10^9/L*	*Hb g/dL*	*Platelets x 10^9/L*
1	HH	51	F	IgG lambda	No	Yes	12.2	8.3	618
2	SKH	67	M	IgG kappa	No	Yes	7.1	12.3	310
3	PL	62	M	IgG kappa	No	No	8.4	12.6	323
4	SM	59	M	Light chain kappa	No	Yes	8.5	8.5	468
5	KC	48	M	IgG lambda	Yes	Yes	7.9	7.6	238
6	GB	55	F	Light chain lambda	Yes	Yes	5.3	7.1	102
7	SU	65	F	Light chain lambda	Yes	Yes	6.0	8.0	399
8	KI	47	F	IgG lambda	No	Yes	16.1	14.7	411
9	SK	59	M	IgG kappa	Yes	Yes	6.0	9.5	102

Abbreviations in Tables 6 and 7: F: female; FAB: French-American-British classification; Hb: hemoglobin; M: male; Ig: immunoglobulin; RA: refractory anemia; RAEB: refractory anemia with excess of blasts; RARS: refractory anemia with ringed sideroblasts; WBC: white blood cells.

2.2 Culture media

Iscove's modified Dulbecco's medium (IMDM) 350

17.66g	IMDM	Invitrogen Coorperation
10 ml	Penicillin-streptomycin	Invitrogen Coorperation
35 ml	Sodium bicarbonate	Sigma
805 ml	H_2O	

Long-term bone marrow culture medium

800 ml	IMDM 350	
100 ml	Fetal Bovine Serum (FBS#06450)	StemCell Technologies
100 ml	Horse Serum (HS#06750)	StemCell Technologies
5×10^{-7} M	Hydrocortisone	Sigma

M2-10B4 medium

85 ml	RPMI1640	Sigma
15 ml	Fetal Calf Serum (FCS A15-043)	PAA Laboratories
1 ml	10,000 U/ml Penicillin-streptomycin	Invitrogen Coorperation
2 ml	200 mM L-Glutamine	Invitrogen Coorperation

Fibroblast medium

85 ml	IMDM350	
15 ml	Fetal Bovine Serum (FBS#06472)	StemCell Technologies

Methylcellulose and growth factors for colony assays

80 ml	MethylCult H4230	StemCell Technologies
0.03ug/ml	rh GM-CSF	PeproTech
0.03ug/ml	rh G-CSF	PeproTech

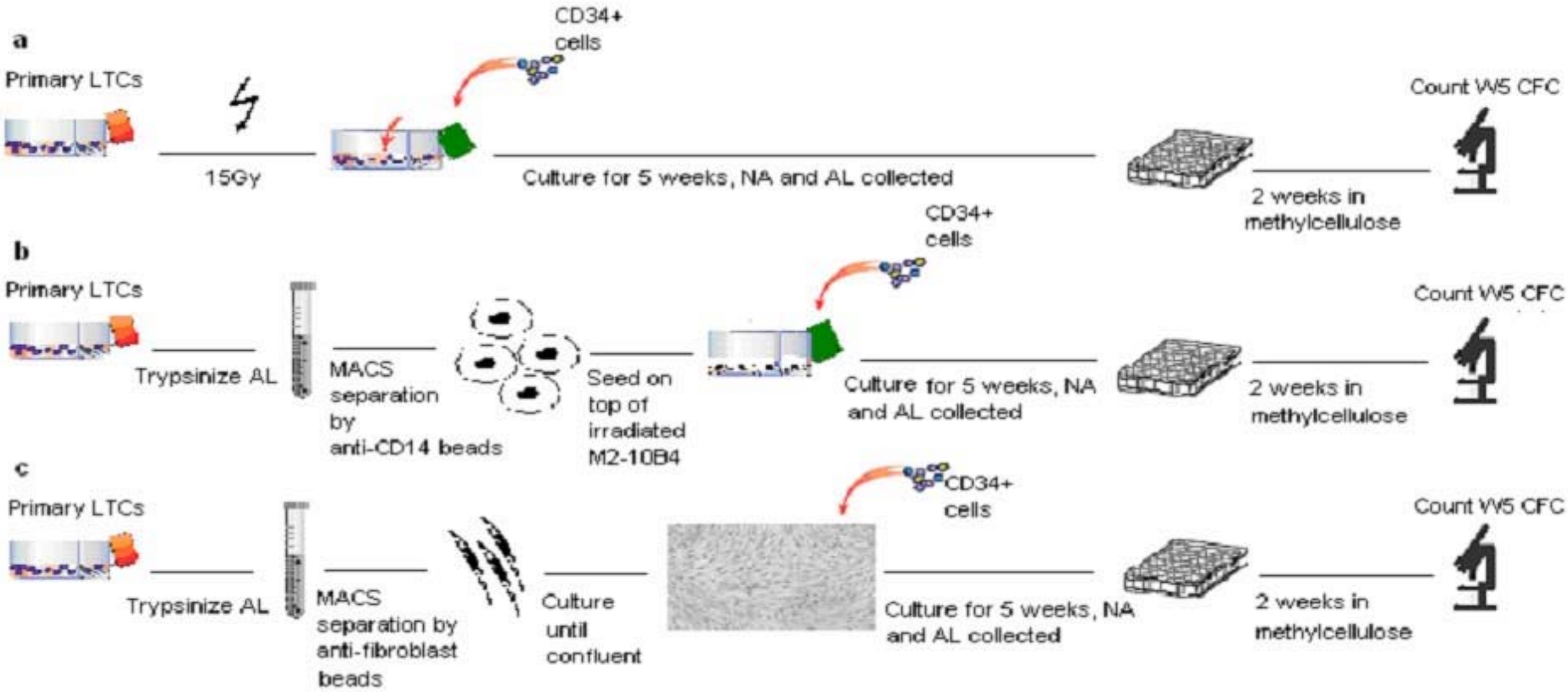

Fig 1. Experimental design for the study of the hematopoiesis-supporting ability of the intact stroma layer (panel a, depletion of endogenous hematopoietic cells by irradiation), isolated stroma macrophages co-cultured with irradiated murine M2-10B4 cells (panel b) or isolated stroma fibroblasts (panel c). The hematopoiesis-supporting ability was quantified by enumerating the number of week 5 colony-forming cells (W5 CFC) generated by exogenously added normal CD34+ cells. AL: adherent layer; LTC: long-term culture; MACS: magnetic cell separation; NA: non-adherent cells.

2.3 Magnetic cell separation (MACS) equipment and other reagents

MACS beads

CD14 MicroBeads	Miltenyi Biotec
Anti-Fibroblast MicroBeads	Miltenyi Biotec
Direct CD34 progenitor cell isolation kit	Miltenyi Biotec

MACS instruments

MACS large cell separation columns	Miltenyi Biotec
MACS magnetic field	Miltenyi Biotec

MACS buffer

95 ml	Phosphate-buffered saline (PBS)	Gibco
5 ml	Bovine serum albumin (BSA)	StemCell Technologies
2 mM	EDTA	Sigma

Other reagents

Ammonium chloride lysing reagent	PharMingen
Trypsin / EDTA (10×)	PAA Laboratories
MethylCult H4100	StemCell Technologies

2.4 Cell cultures

2.4.1 Primary long-term bone marrow cultures

Bone marrow samples were obtained by aspiration from the posterior iliac crest and collected in heparin-containing tubes. Samples were processed within four hours after aspiration. The marrow was diluted 1 : 1 with PBS, mixed with methylcellulose at a final concentration of 0.1% and placed at room temperature for 35-40 minutes to promote red cell separation by gravity sedimentation. The top layer containing the nucleated cell fraction was collected, washed once in PBS and subjected to erythrocyte lysis using ammonium chloride lysing reagent. The cells were then seeded at a density of 2×10^6 cells per ml in 5 ml long-term bone marrow culture medium in T 12.5 cm^2 tissue culture flasks (Coutinho et al., 1986). Parallel cultures were prepared to obtain sufficient numbers of adherent cells. For the first week, the

cultures were incubated at 37°C in a 95% humidified atmosphere of 5% CO_2 in air and then transferred to 33°C for another 2-3 weeks until a confluent adherent layer had formed. The cultures were maintained by weekly demi-depopulation and substitution of half the spent medium by an equal volume of fresh medium (Coutinho et al., 1986).

2.4.2 Two-stage long-term bone marrow cultures

The entire experimental procedure is shown in Fig. 1. Details of the methods are described below.

2.4.2.1 Establishment of an adherent stroma layer

After three to four weeks of cultivation, most cultures developed a confluent stroma layer. Confluency of cultures was evaluated under the inverted microscope by dividing the flask bottom into twenty equal squares and counting the proportion of squares occupied by the adherent layer. Cultures with a confluency of more than 15% were used for the subsequent steps.

2.4.2.2 Depletion of endogenous hematopoietic cells by irradiation of the stroma layer

In one part of the study, one of the culture flasks from each marrow sample was exposed to 15 Gy irradiation (250 kV peak x-ray) to eliminate endogenous benign and malignant hematopoietic cells. The supernatant was subsequently replaced by fresh long-term bone marrow culture medium (Coutinho et al., 1986).

2.4.2.3 Fractionation of the stroma by immunomagnetic cell separation

In another part of the study, one or more flasks from each marrow sample were used for the isolation of stroma fibroblasts and macrophages using immunomagnetic techniques. Confluent stroma was washed thoroughly with PBS to eliminate non-adherent cells, and then detached by adding 1.5 ml of a 0.25% trypsin/PBS solution followed by incubation in 37°C for 5 minutes and addition of an equal volume of ice-cold FCS to neutralize trypsinization. In order to obtain a single cell suspension without cell clumps, the sheets of detached adherent cells were passed through a 30 µm nylon mesh followed by vigorous pipetting. Cells were counted using a hemocytometer, and aliquots of 10^7 cells were resuspended in 80 µl buffer.

2.4.2.4 Establishment of a pure fibroblast stroma layer

20 µl MACS anti-fibroblast microbeads were added to 10^7 trypsinized stroma cells suspended in 80 µl buffer and incubated for 30 minutes at 20° to 25°C. Subsequently, the cells were passed through a MACS large cell separation column placed in a magnetic field. Antibody-labelled cells were detained in the column while unlabelled cells passed through. Positive cells were collected by removal of the column from the magnetic field and flushing with buffer. To evaluate the purity of the isolated cell populations cytospins were prepared and stained with May-Grünwald-Giemsa. Stroma fibroblasts were identified as cells with large irregular euchromatic nuclei and blue cytoplasm. Stroma macrophages were identified as cells with small round heterochromatic nuclei and vacuolated cytoplasm. Only separations with more than 92% purity were used for the subsequent steps in the experimental protocol. The isolated stroma fibroblasts were cultured in fibroblast medium at 37°C in a 95% humidified atmosphere of 5% CO_2 in air until reaching confluency.

2.4.2.5 Establishment of a stroma layer composed of macrophages and M2-10B4 cells

Since macrophages fail to form a confluent adherent layer and are unable to support long-term hematopoiesis in the absence of other stroma cell types, the effect of macrophages on the performance of the microenvironment was analysed by co-culturing isolated human stroma macrophages with murine M2-10B4 cells which have been shown to support human hematopoiesis in vitro (Sutherland et al., 1991). M2-10B4 cell cultures without added macrophages served as a control in these experiments.

In order to obtain pure stroma macrophages 20 µl MACS CD14 microbeads were added to 80 µl of a suspension containing 10^7 detached stroma cells. After incubation at 6° to 12°C for 15 minutes, the cells were passed through a MACS large cell separation column placed in a magnetic field. Antibody-labelled cells were detained in the column while CD14-negative cells passed through. Positive cells were collected by removal of the column from the magnetic field and flushing with buffer. Cytospins were prepared to evaluate the efficacy of the fractionation procedure. The isolated stroma macrophages were plated on top of irradiated M2-10B4 cultures at a concentration of 3×10^5 cells per 5 ml long-term bone marrow culture medium in a T 12.5 cm^2 flask.

M2-10B4 cells were maintained in T 12.5 cm^2 tissue culture flasks in M2-10B4 medium and passaged once they got confluent. To this end old medium was decanted, confluent stroma

was washed once with PBS, and 1.5 ml of a 0.25% trypsin/PBS solution was added. After an incubation period of 5 minutes at 37°C the enzymatic reaction was stopped by addition of 1.5 ml of ice-cold FCS. Cells were washed once and counted. 4×10^5 cells were resuspended in 5 ml of M2-10B4 medium, transferred to a new T 12.5 cm^2 flask and incubated for 3 days at 37°C in a 95% humidified atmosphere of 5% CO_2 in air to achieve confluency. In order to create comparable conditions for each set of experiments several flasks were prepared in parallel. To prevent overgrowth and stop cell division confluent M2-10B4 cultures were irradiated with 30 Gy using the same apparatus as used for human long-term cultures. After irradiation the M2-10B4 medium was replaced by long-term bone marrow culture medium.

2.4.2.6 Purification of CD34+ indicator cells

Human CD34+ cells served as indicator cells for the hematopoiesis-supporting function of the stroma and its isolated cellular constituents. CD34+ cells were obtained with informed consent from excess leukapheresis material from a patient with multiple myeloma undergoing autologous stem cell transplantation. CD34+ cells were selected by MACS separation using a direct CD34 progenitor cell isolation kit. In principal, separation followed the same protocol as described for the isolation of fibroblasts and macrophages. The purity of the separated cells as assessed by fluorescence-activated cell analysis was more than 96% (data not shown). Aliquots of cells were preserved in liquid nitrogen for long-term use.

2.4.2.7 Co-culture of CD34+ cells with a pre-established adherent layer

2.5×10^4 CD34+ cells were seeded onto different types of pre-established stroma layers including unfractionated irradiated long-term culture layers, pure fibroblast layers, irradiated M2-10B4 layers alone and irradiated M2-10B4 layers with purified human stroma macrophages. The co-cultures were maintained in T 12.5 cm^2 tissue culture flasks in 5 ml long-term bone marrow culture medium at 33°C in a 95% humidified atmosphere of 5% CO_2 in air for 5 weeks with weekly half-medium change as described above. After 5 weeks of co-culture, both non-adherent and adherent cells were collected, counted and plated in methylcellulose cultures for the enumeration of granulocyte-macrophage colony-forming cells (CFC). The sum of the CFC in the non-adherent and adherent cell fractions at week 5 were termed W5 CFC.

2.4.2.8 Colony assays in methylcellulose cultures

An appropriate number of cells harvested from long-term cultures were mixed with 1 ml of G-CSF and GM-CSF supplemented 2.6% methylcellulose medium, plated in triplicates in 24-well plates and incubated for 14 days at 37°C in a 95% humidified atmosphere of 5% CO_2 in air. On day 14, colonies (defined as cell aggregates of more than 40 cells) were scored using an inverted microscope.

2.5 Statistical analysis

A non-parametric Mann-Whitney U test was used to compare W5 CFC values between groups. Wilcoxon analysis was used to compare paired samples. Correlation analysis was assessed using Spearman analysis for asymmetrically distributed samples. P values below 0.05 were considered to demonstrate statistically significant results. All calculations were done using the SPSS 10.0 software (SPSS, Chicago, IL, USA).

3 Results

3.1 Stroma function in acute myeloid leukemia

3.1.1 Primary long-term bone marrow cultures

Confluent stroma was obtained after three to four weeks of culture. There was no statistically significant difference in the degree of confluency between normal (70 ± 28%, range 30 - 100%) and AML stroma (61 ± 31%, range 15 - 100%). However, subtle morphological differences were observed under the inverted microscope as shown in Figure 2. In normal long-term cultures, abundant adipocytes and ample cobble stone area formation were seen representing the growth of stem and early progenitor cells in the stroma layer. By contrast, adipocytes were scarcely seen in the stroma of AML cultures.

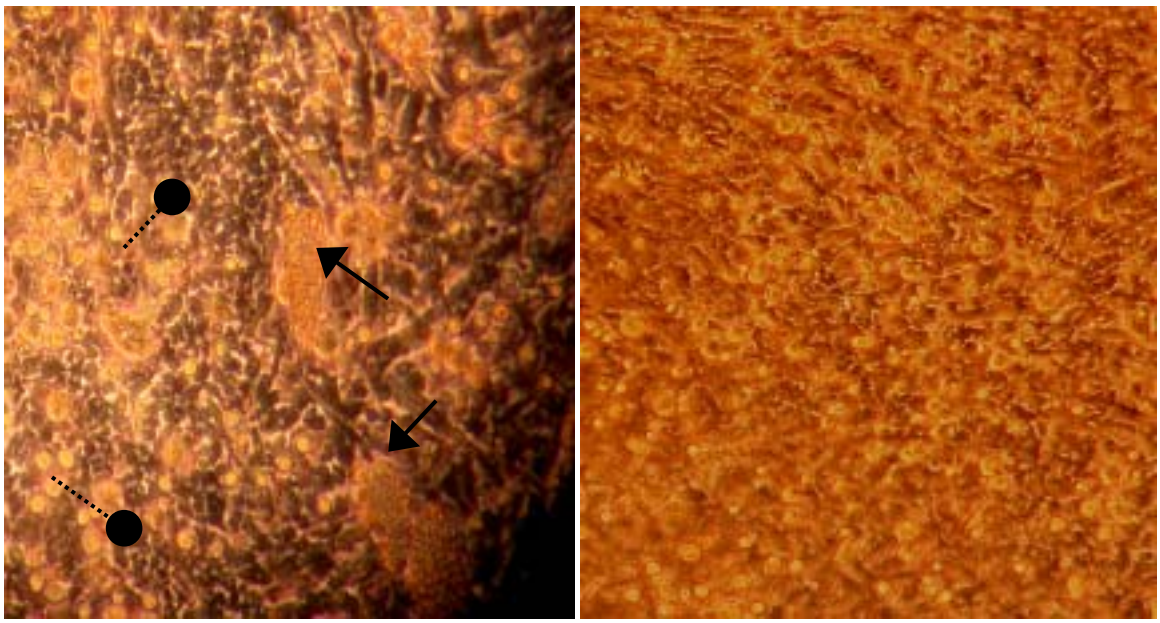

Fig. 2: Primary long-term culture stroma from a normal individual (left) and an AML patient (right). The left picture shows abundant adipocytes (arrows) besides patches of cobble stone areas (solid circles with dotted lines), while the right picture shows no adipocytes and few hematopoietic foci although the stroma was fully confluent. (Phase-contrast inverted microscope, original magnification *100)

The hematopoiesis-supporting function of the stroma was assessed by counting the number of nucleated cells removed during weekly demi-depletion of the cultures and determining the number of colony-forming cells in G-CSF and GM-CSF stimulated methylcellulose cultures. Figure 3a shows the behavior of total nucleated cells in the supernatant of the cultures. AML cultures were clearly different from normal cultures showing two different growth patterns. In pattern 1 (2 patients) cell numbers were persistently higher than in the control group. In

pattern 2 (4 patients) the cell numbers remained in the same range as in the control cultures during the first four or five weeks, with a rapid decline below the normal range at later time points. While in the control group all curves showed a plateau after the first three weeks of culture, a continuous decline until the end of the culture period was observed in the group of AML patients displaying pattern 2.

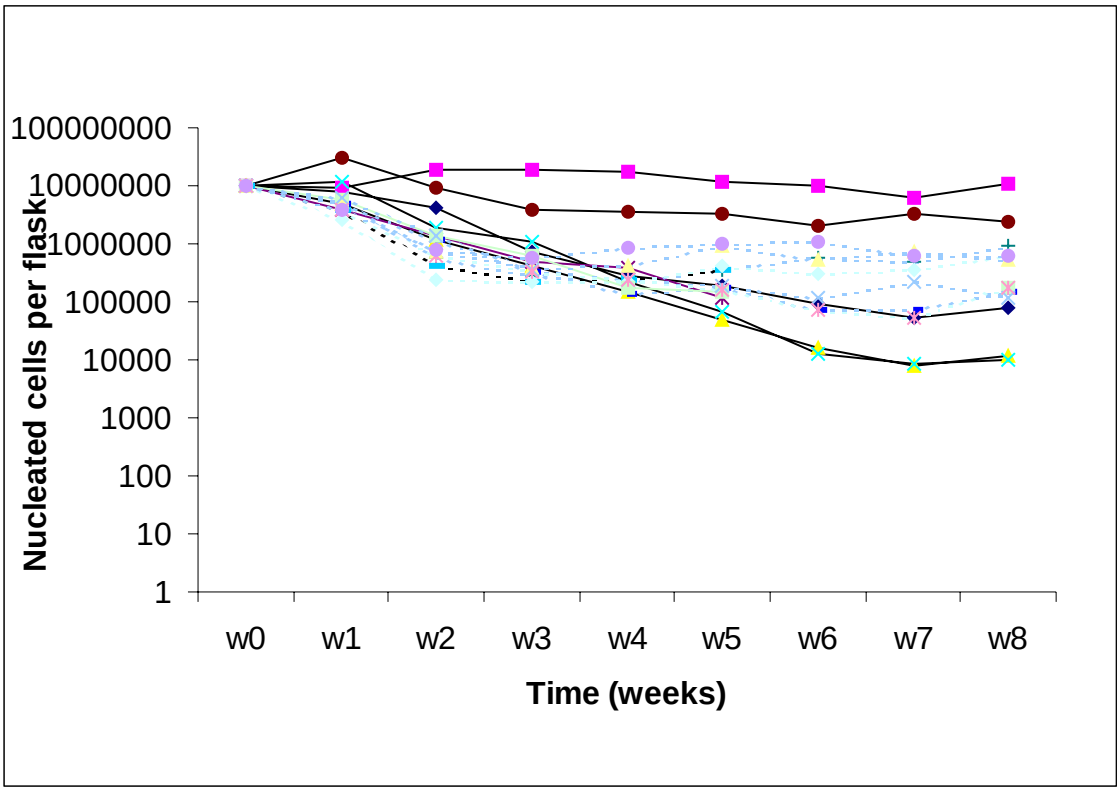

Fig. 3a: Numbers of nucleated cells in the supernatant of long-term bone marrow cultures from 6 AML patients (solid lines) and 8 controls (dotted lines) during an 8-week incubation period.

Granulocyte-macrophage colonies were enumerated according to established criteria. In the methylcellulose cultures from some AML patients morphologically abnormal colonies or clusters (i.e. cell aggregates of fewer than 40 cells) were observed. These colonies or clusters were thought to originate from the proliferation of leukemic blasts and made an accurate enumeration of normal colonies impossible. Figure 3b shows the weekly output of granulocyte-macrophage colony-forming cells (CFC) in the supernatant of control and AML cultures. Again, the AML group displayed two different growth patterns. Pattern 1 showed a persistently higher output of CFC compared with the control cultures. Pattern 2 showed a sharp decline after the second week of cultivation with few if any CFC remaining thereafter. In the methylcellulose cultures of the two patients with the first pattern, most colonies and clusters showed an abnormally diffuse morphology and their size was highly variable,

suggesting that they represented the growth of leukemic blasts. In such cultures colonies of normal morphology were virtually not observed.

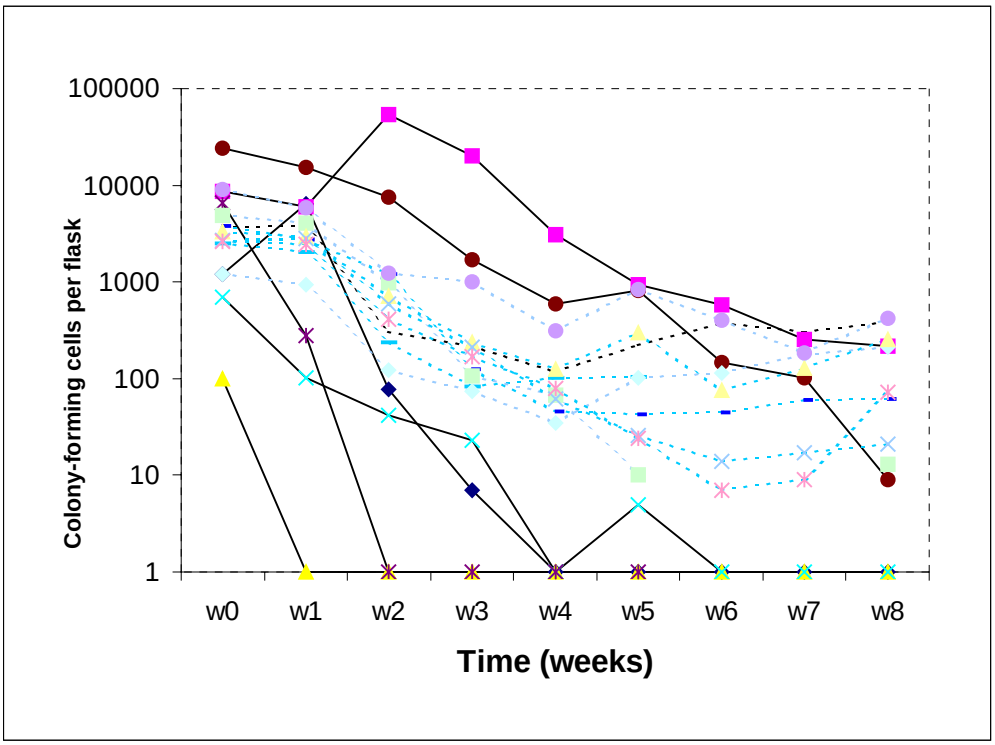

Fig. 3b: Numbers of colony-forming cells in the supernatant of long-term bone marrow cultures from 6 AML patients (solid lines) and 8 controls (dotted lines) during an 8-week incubation period.

The colony-forming cells in the non-adherent cell fraction are considered to be less good a parameter for the hematopoiesis-supporting function of the stroma than the colony-forming cells in the adherent layer which contains the most primitive precursors. A good quantitative measure for the overall capacity of the stroma to support blood cell formation is the combined output of colony-forming cells in the non-adherent cell fraction and the adherent layer after a 5-week culture period (week 5 colony-forming cells, W5 CFC). Figure 4 shows W5 CFC numbers from cultures of AML patients and controls. If one excludes the two patients with high W5 CFC numbers which were likely to originate from leukemic blasts, significantly decreased W5 CFC numbers were observed in AML cultures in comparison to control cultures ($P = 0.009$).

In principal, decreased numbers of W5 CFC can be related to decreased numbers of stem cells in the initial inoculum or to a decreased hematopoiesis-supporting function of the stroma. The following experiments were designed to detect abnormalities in the bone marrow stroma of patients with acute myeloid leukemia.

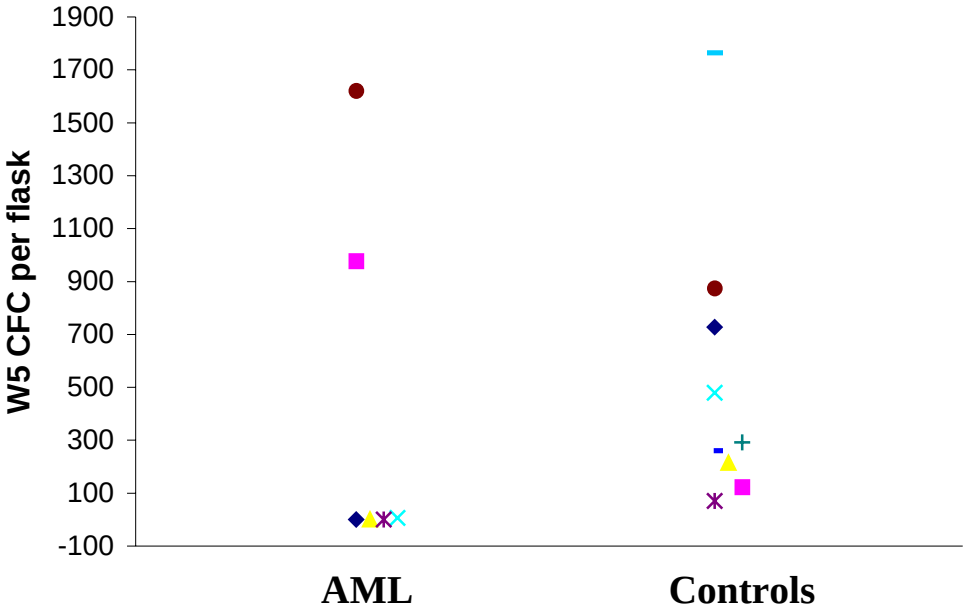

Fig. 4: Numbers of week 5 colony-forming cells (W5 CFC) in long-term bone marrow cultures from 6 AML patients and 9 controls.

3.1.2 Two-stage long-term bone marrow cultures

3.1.2.1 Hematopoiesis-supporting function of unfractionated bone marrow stroma

When the adherent layer of the long-term cultures reached confluency, the flasks were irradiated with 15 Gy to eliminate endogenous blood cell formation. Then 2.5 x 10^4 purified CD34+ indicator cells were seeded on top of the irradiated stroma layer, and W5 CFC were determined as a measure of the hematopoiesis-supporting ability of the stroma.

Figure 5 shows W5 CFC derived from AML and control two-stage cultures. In each of the two groups, the values obtained showed a significant degree of heterogeneity. There was no statistically significant correlation between the W5 CFC values and patient age, white blood cell count, cytogenetic abnormalities, stroma confluency or duration before complete remission (P = 0.069, 0.14, 0.15, 0.14 or 0.28, respectively). Since a considerable overlap was observed in the values obtained for AML versus control cultures, there was also no statistically significant difference in the W5 CFC numbers of the two patient groups (P = 0.1).

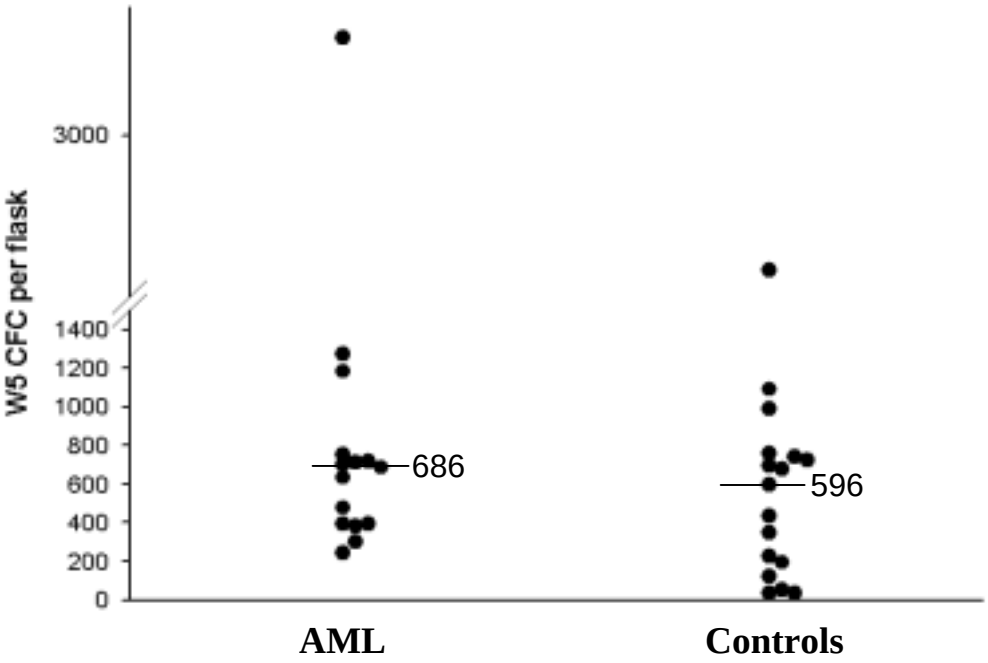

Fig. 5: Numbers of week 5 colony-forming cells (W5 CFC) in two-stage long-term bone marrow cultures from 15 AML patients and 17 controls. After reaching confluency, the cultures were irradiated with 15 Gy and overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

In view of its multicellular composition a normal overall stroma function does not necessarily mean that the function of its individual components is also unperturbed. To address this question the major stroma cell types, fibroblasts and macrophages, were isolated using immunomagnetic beads and analysed separately using W5 CFC output as an indicator for the hematopoisis-supporting ability of the cells.

3.1.2.2 Fractionation of long-term bone marrow culture stroma

Figure 6a shows a cytospin preparation of unfractionated trypsinized long-term culture stroma. The major discernible cell types are fibroblasts and macrophages. Figure 6b shows purified stroma macrophages and fibroblasts after immunomagnetic cell separation using the MACS device. The purity of the isolated cell populations was generally between 90 and 100% (Table 8).

Table 8: Purity of fibroblasts and macrophages from trypsinized long-term culture stroma after immunomagnetic cell separation

Population	Macrophages (n=37)	Fibroblasts (n=37)
Purity	96.5 ± 2.5%	95.0 ± 3.0%

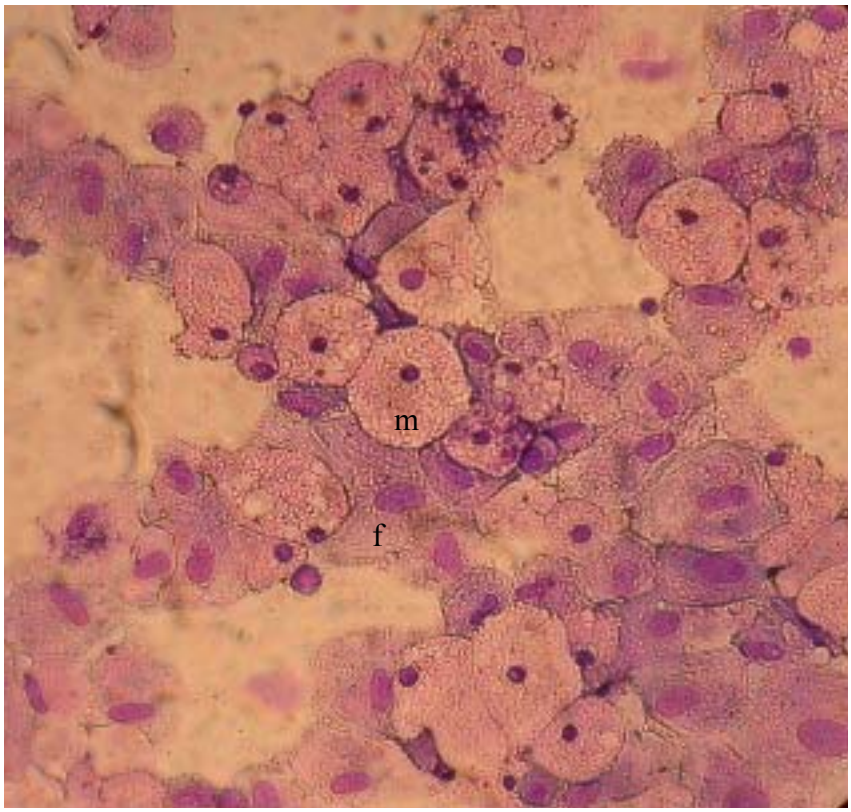

Fig. 6a: May-Grünwald-Giemsa stained cytospin of unfractionated long-term bone marrow culture stroma from a MDS patient. Cells with round heterochromatic nuclei and vacuolated cytoplasm are macrophages (marked "m"), while those with larger irregular euchromatic nuclei and blue cytoplasm are fibroblasts (marked "f").
Original magnification *200.

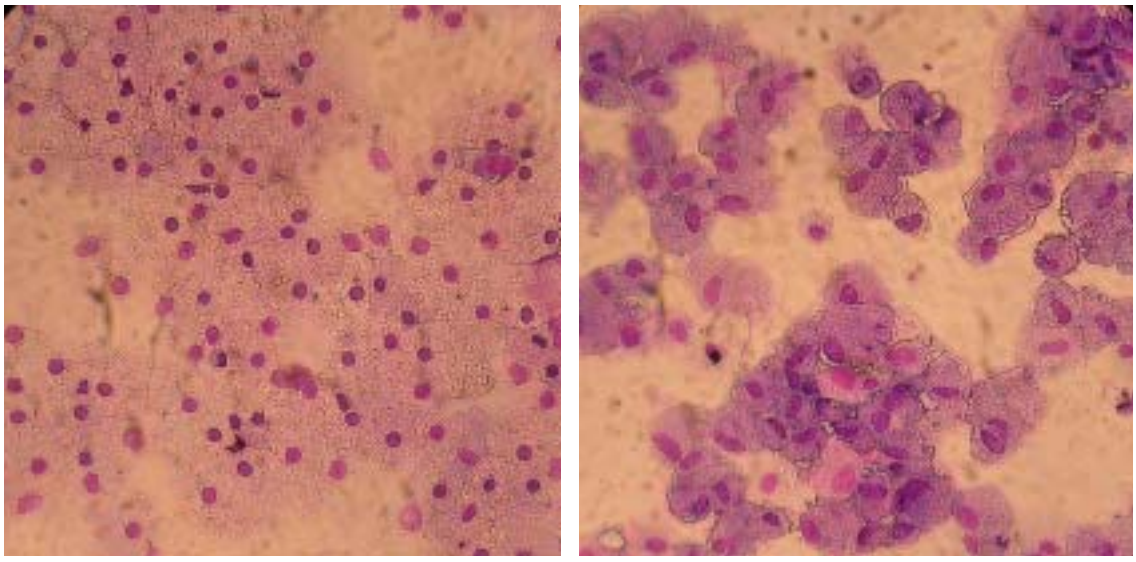

Fig. 6b: May-Grünwald-Giemsa stained cytospins of purified macrophages (left) or fibroblasts (right), respectively, from long-term bone marrow culture stroma. Original magnification *200.

3.1.2.3 Hematopoiesis-supporting function of stroma fibroblasts

The bone marrow aspirates from 14 patients with AML and 11 controls were of sufficient quantity to allow the preparation of the required number of long-term cultures for stroma cell fractionation studies. Cell fractionation was not possible in patient no. 5 (RA) of the AML group and in patients no. 1 (SM), 3 (WKD), 12 (BM), 13 (RI), 15 (VL) and 17 (DB) of the controls (cf. Tables 3 and 5).

After immunomagnetic separation fibroblasts were seeded in new culture flasks, allowed to reach confluency, and then overlaid with 2.5×10^4 normal CD34+ cells. W5 CFC numbers were used as a measure of the hematopoisis-supporting ability of the adherent layer. Confluency of the fibroblast layer was reached by the cells from all but one patient (AML patient no. 3 (DK), cf. Table 5). The hematopoiesis-supporting ability of this culture (310 W5 CFC), however, was in the same range as that of the other AML samples (131 – 1723).

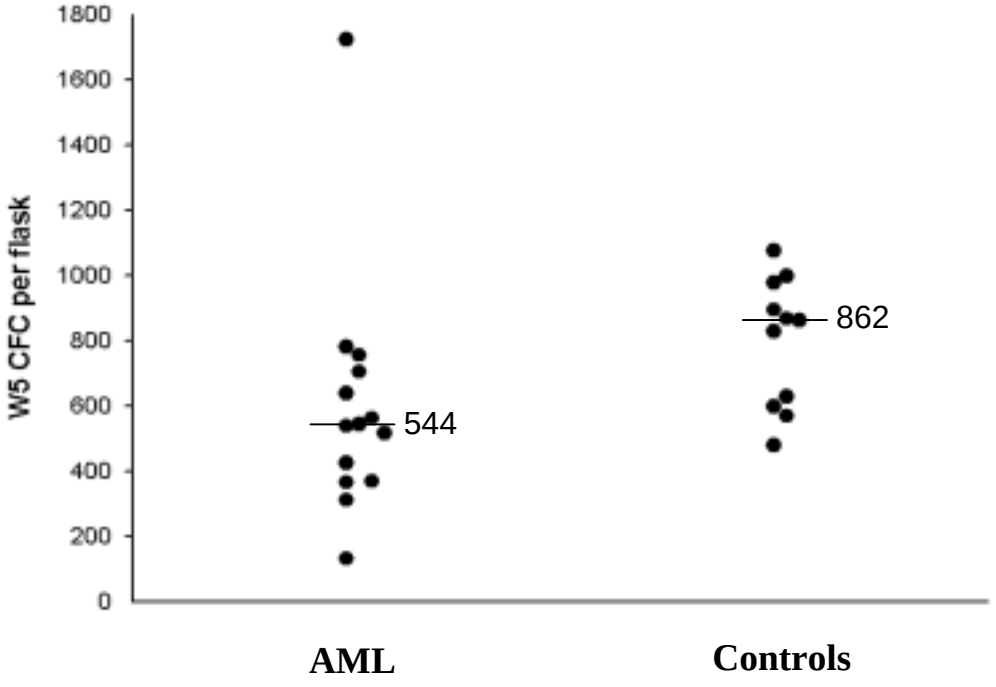

Fig. 7: Numbers of week 5 colony-forming cells (W5 CFC) in pure stroma fibroblast cultures from 14 AML patients and 11 controls. After reaching confluency, the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

Figure 7 shows W5 CFC numbers in two-stage cultures supported by purified fibroblasts from AML and control long-term bone marrow cultures. Although considerable heterogeneity was observed in both groups with respect to the numbers of W5 CFC, on the average AML fibroblasts showed a significantly lower capacity to support the growth of

CD34+ cells than the fibroblasts from the controls (P = 0.018, Mann-Whitney U test). There was no correlation between the hematopoisis-supporting function of purified fibroblasts and the patient's age, cytogenetic abnormalities, the degree of confluency of the adherent layer or the performance of the corresponding unfractionated adherent stroma layer.

3.1.2.4 Hematopoiesis-supporting function of stroma macrophages

An assessment of the hematopoiesis-supporting function of purified stroma macrophages requires different conditions from those used for stroma fibroblasts. Stroma macrophages are less frequent than stroma fibroblasts, they are virtually non-dividing failing to form a confluent adherent layer, and, if plated at low density, may be rapidly overgrown by small numbers of contaminating fibroblasts. To circumvent problems related to these properties purified macrophages were co-cultured with a confluent layer of irradiated murine M2-10B4 cells which are known to support human hematopoiesis (Sutherland et al., 1991). These co-cultures were then overlaid with 2.5×10^4 normal CD34+ indicator cells, and week 5 colony-forming cells were determined to measure the hematopoiesis-supporting activity of the cultures.

During the course of the experiments two different batches of M2-10B4 cells were used. The first batch used represented late passage cells that had been cultured in our laboratory for various purposes for several years. These cells showed slow growth, abnormal morphology on May-Grünwald-Giemsa-stained cytospins (large size, vacuolated cytoplasm, fragmented nucleus) and poor support of human CD34+ cells. Whether these properties were related to the age of the cell line or outgrowth of an abnormal subclone, remained unresolved. In order to improve the culture conditions a new cryopreserved batch was thawed and used in all subsequent experiments. The cells from this batch showed rapid growth and good support of CD34+ cells. In the subsequent description of the experiments, the first batch will be referred to as 'late passage' and the second one as 'early passage' M2-10B4 cells. The results obtained with the two types of cells are reported separately. Because in conjunction with macrophages early passage M2-10B4 cells were quite consistently about 9 times more active than late passage M2-10B4 cells, the results obtained with the two batches are also presented together by multiplying the values generated with late passage cells by 9.

Figure 8a shows W5 CFC data obtained with late passage M2-10B4 cells. Although 55% of the AML cultures showed W5 CFC numbers above the range of the controls and the median

CFC numbers in the AML and control groups differed by more than two-fold (210 versus 98), these differences failed to reach statistical significance.

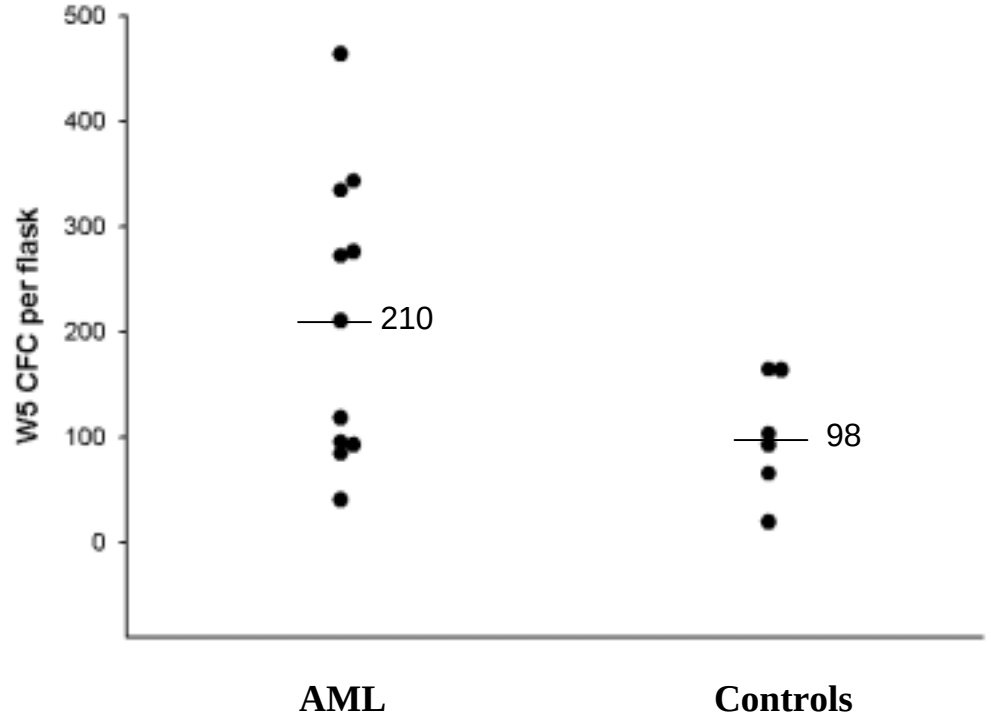

Fig. 8a: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of 'late passage' M2-10B4 cells with stroma macrophages from 11 AML patients and 6 controls. The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

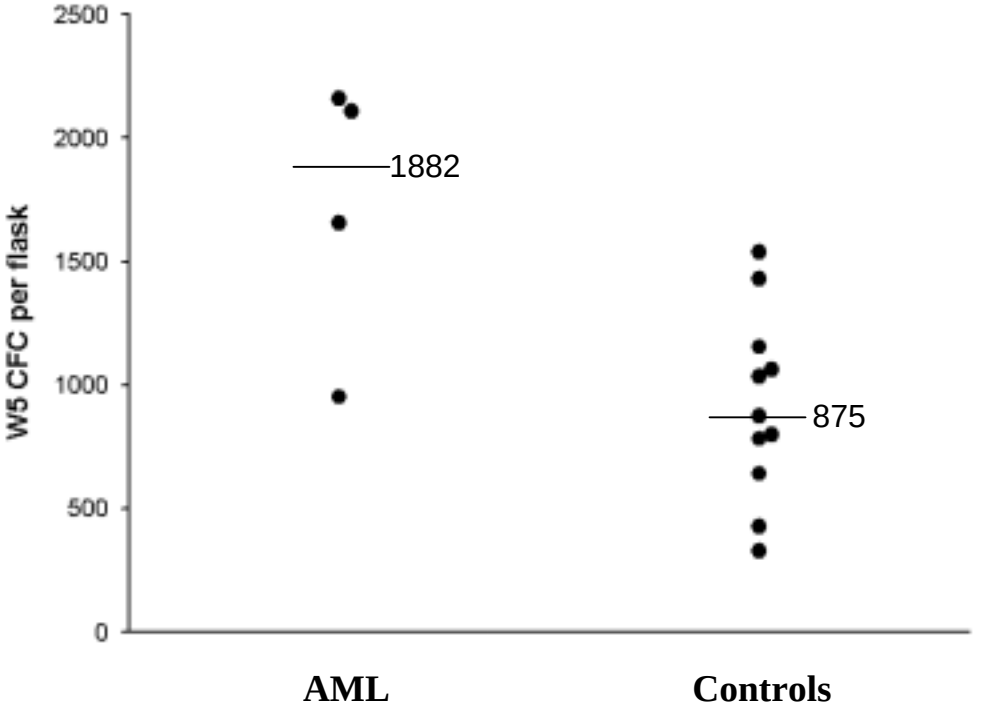

Fig. 8b: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of 'early passage' M2-10B4 cells with stroma macrophages from 4 AML patients and 11 controls. The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

Figure 8b shows the W5 CFC data obtained with early passage M2-10B4 cells. Similar to the findings with late passage cells, 75% of the values in the AML group were above the range in the control group with a median of 1882 versus 875. In this set of experiments, statistical significance was demonstrated using the Mann-Whitney U test (P = 0.026).

The ratio between the median W5 CFC numbers observed in the set of experiments using early passage M2-10B4 cells and the set of experiments using late passage M2-10B4 cells was almost identical in the AML group (8.96) and the control group (8.93). Assuming that early passage M2-10B4 cells are about 9 times more active than late passage M2-10B4 cells we multiplied the results obtained with late passage cells by this factor. Figure 8c shows the combined data of the two experimental periods in one graph. The combined set of data showed a statistically highly significant difference between the AML group and the control group (P = 0.008). Stroma macrophages from AML patients were significantly better supporters of hematopoiesis than stroma macrophages from individuals with a healthy bone marrow. This conclusion was confirmed by the aspect of the cultures under the inverted microscope. Cobble stone areas were more frequent and larger in M2-10B4 cultures containing AML macrophages than in cultures containing normal macrophages (Fig. 9).

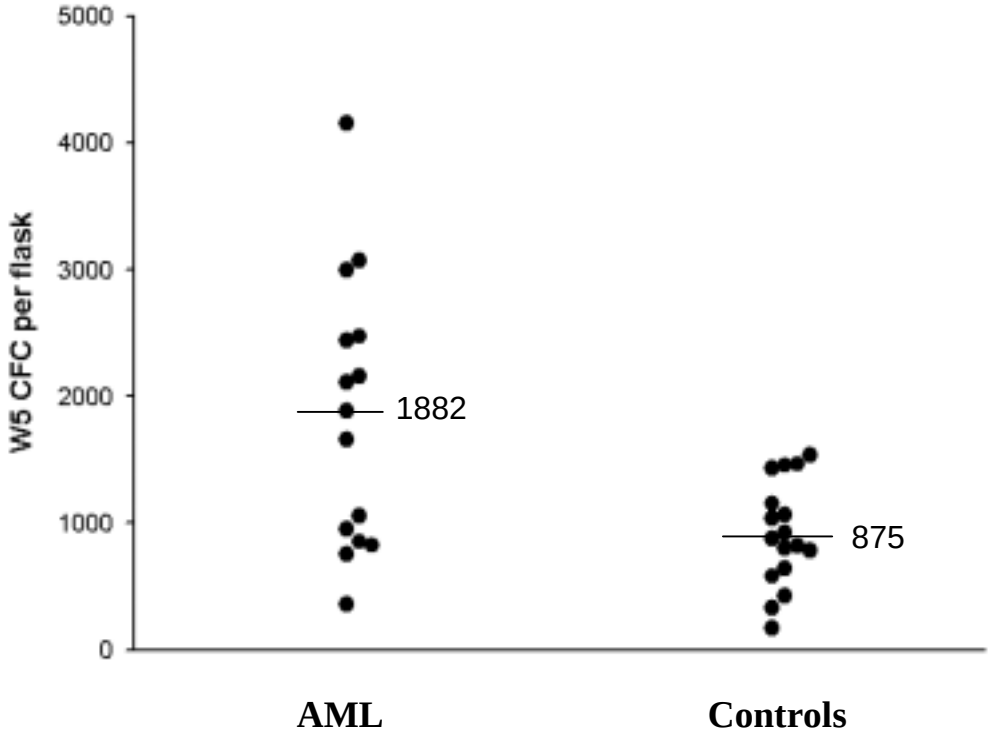

Fig. 8c: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of 'early' or 'late passage' M2-10B4 cells with purified stroma macrophages from 15 AML patients and 17 controls. The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. The data generated with cultures containing 'late passage' M2-10B4 cells were recalculated as described in the text. Lines represent median values.

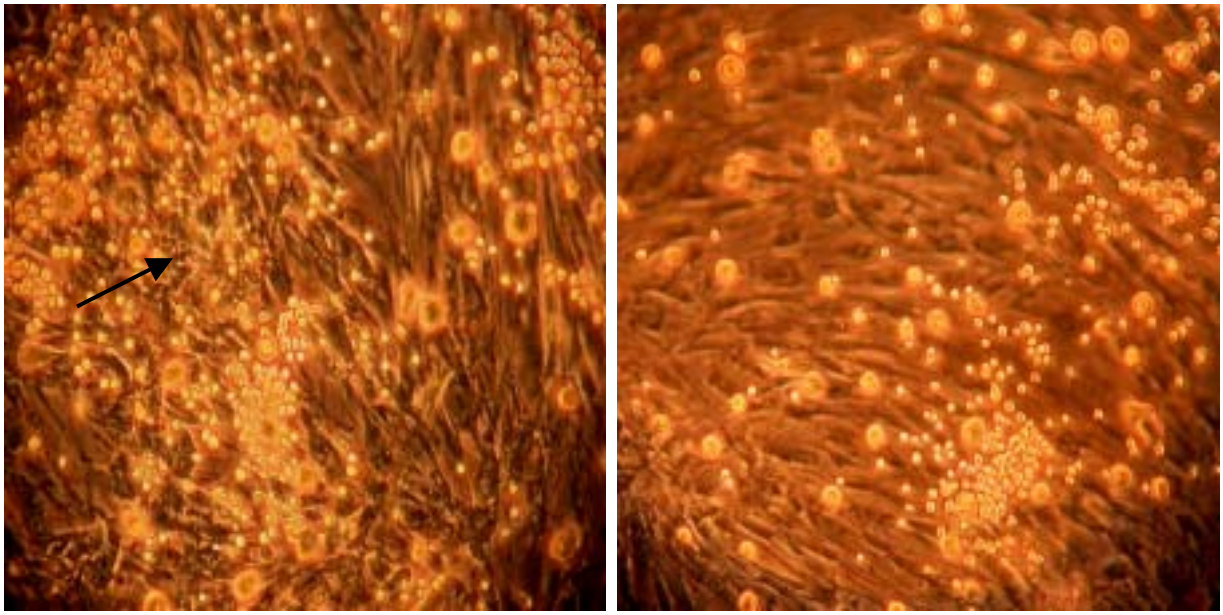

Fig. 9: Co-cultures of M2-10B4 cells and purified stroma macrophages from a patient with AML (left panel) and a control individual with a healthy bone marrow (right panel). The cultures were overlaid with 2.5×10^4 CD34+ cells and incubated for 5 weeks. M2-10B4 cells cover the bottom of the culture vessel as spindle-like structures, macrophages are large round cells, and maturing hematopoietic cells can be seen on top of the adherent layer as small shiny cells. The culture containing AML macrophages shows more abundant hematopoietic cells and, in addition, a small cobble stone area (very small dark cells within the adherent layer; arrow).
Phase-contrast inverted microscope, original magnification *100.

In an effort to relate the behavior of stroma macrophages to other variables correlation analysis was performed testing the following factors: age, cytogenetic abnormalities, hematopoiesis-supporting ability of whole stroma and purified stroma fibroblasts, and duration before complete remission. No statistically significant correlations were observed.

In order to exclude the possibility that the variability observed within the groups of AML patients and controls was related to technical problems occurring in the course of the complex cell culture procedure, parallel experiments were performed in two patients. A large bone marrow sample was used to prepare multiple primary long-term cultures. Each flask was handled separately including cell fractionation, co-culture of purified macrophages with M2-10B4 cells and CD34+ cells, and determination of W5 CFC. The results obtained for the two patients are shown in Tables 9a and 9b. The variation from flask to flask was small making technical reasons for the observed variability unlikely.

Table 9a. Reproducibility of the experimental procedure - experiment 1

	Week 5 colony-forming cells		
Flask No.	Supernatant	Adherent layer	Total
1	464	966	1430
2	535	663	1198
3	549	1057	1606
4	585	1002	1584
5	529	943	1472
6	493	990	1483
Mean ± SD	525 ± 42	936 ± 139	1462 ± 146
CV	8%	15%	9%

Table 9b. Reproducibility of the experimental procedure - experiment 2

	Week 5 colony-forming cells		
Flask No.	Supernatant	Adherent layer	Total
1	687	878	1565
2	602	642	1244
3	686	907	1593
4	494	847	1341
Mean ±SD	687 ± 91	819 ± 120	1436 ± 170
CV	13%	15%	12%

SD: standard deviation; CV: constant variation (mean divided by SD)

3.1.2.5 Influence of chemotherapy on stroma function

In order to study the impact of chemotherapy on the hematopoiesis-supporting function of stroma cells, aspirates from 5 patients with AML (no. 1 [WL], no. 2 [TR], no. 6 [ME], no. 11 [ZD] and no. 15 [BV]) were analysed before and after treatment of the patients. The only patient in complete remission at the time of the second analysis was patient no. 11.

Figure 10a shows the function of unfractionated AML stroma before and after chemotherapy. A consistent decline was observed in the capacity of post-chemotherapy stroma to support the production of W5 CFC by normal CD34+ cells (P = 0.043, Wilcoxon test). In four of the 5 patients studied a deterioration of stroma function was also observed when purified stroma fibroblasts were used as a support layer (Fig. 10b). Only the fibroblasts from patient no. 11 showed an improved hematopoiesis-supporting capacity after chemotherapy. This result did not reach statistical significance.

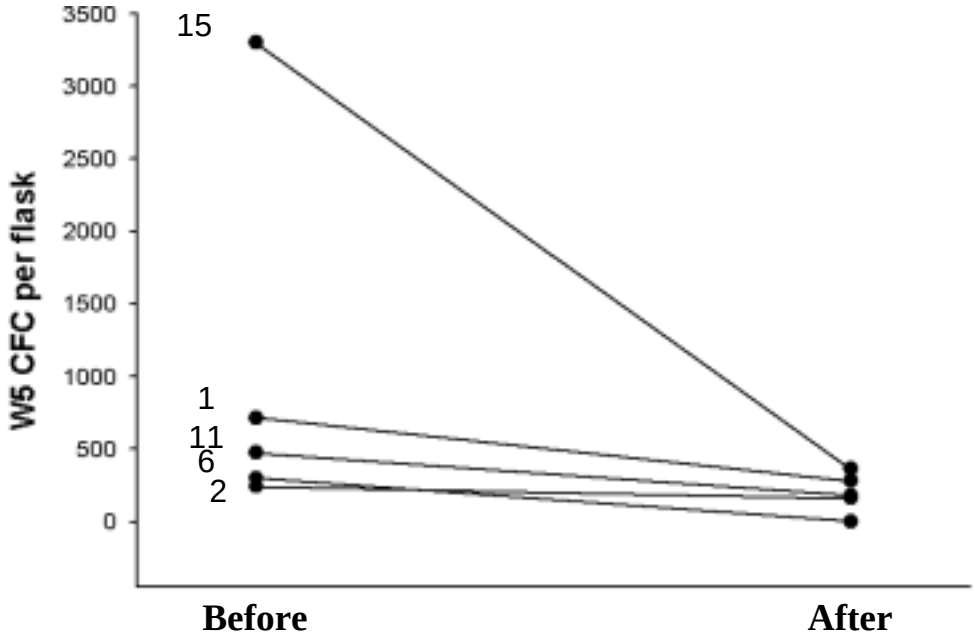

Fig. 10a: Numbers of week 5 colony-forming cells (W5 CFC) in two-stage long-term cultures from 5 AML patients whose bone marrow was analysed both before and after chemotherapy. After reaching confluency, the cultures were irradiated with 15 Gy and overlaid with 2.5×10^4 CD34+ cells. Numbers refer to the patient code used in Table 5.

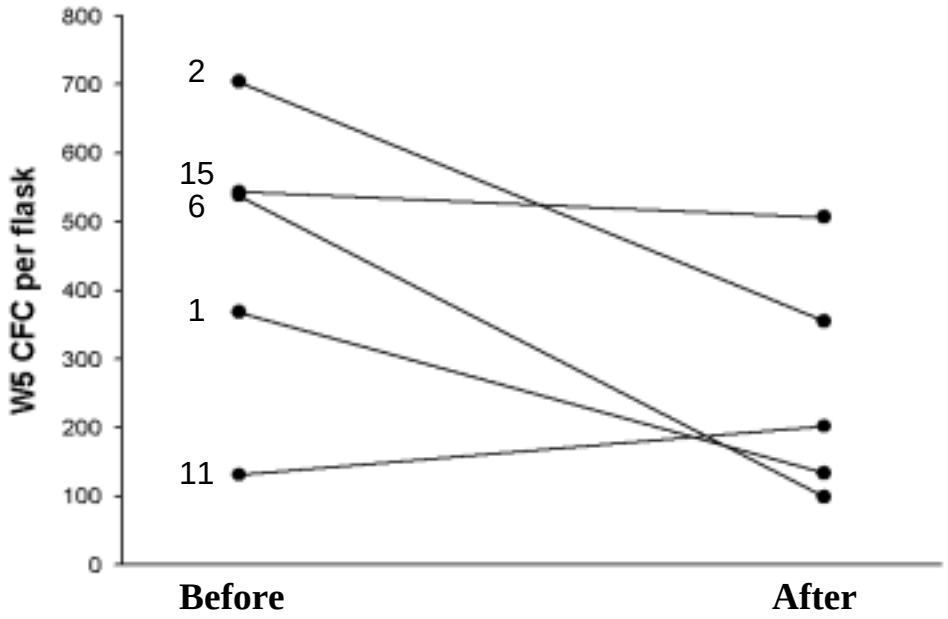

Fig. 10b: Numbers of week 5 colony-forming cells (W5 CFC) in pure stroma fibroblast cultures from 5 AML patients whose bone marrow was analysed both before and after chemotherapy. After reaching confluency, the cultures were overlaid with 2.5×10^4 CD34+ cells. Numbers refer to the patient code used in Table 5.

Because the batch of M2-10B4 cells used for co-culture with macrophages was changed during the course of the experiments, pre- and post-chemotherapy macrophage function could only be compared in three patients (Fig. 10c). In all these cases a considerable decrease in the hematopoiesis-supporting function of the stroma macrophages was observed (P = 0.11).

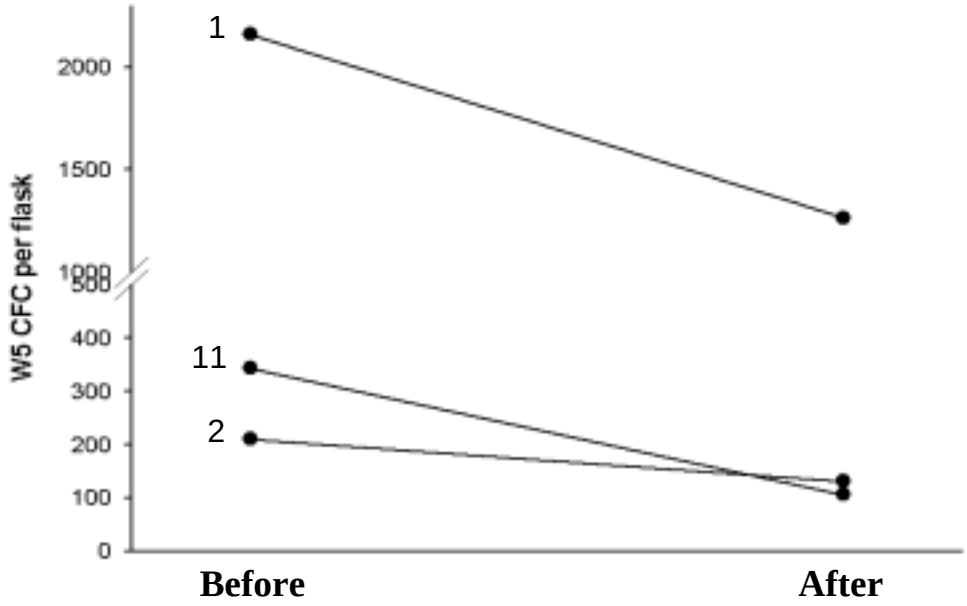

Fig. 10c: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of M2-10B4 cells with stroma macrophages from 3 AML patients whose bone marrow was analysed both before and after chemotherapy. The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. Numbers refer to the patient code used in Table 5.

3.2 Stroma function in myelodysplastic syndromes

3.2.1 Hematopoiesis-supporting function of unfractionated bone marrow stroma

To evaluate the hematopoiesis-supporting function of the entire bone marrow stroma, primary long-term cultures from bone marrow aspirates from 6 MDS patients were grown to confluency, irradiated and reseeded with purified CD34+ cells as described for the AML group. The control cultures were the same as those used in the AML study. The average confluency of the adherent layer in the MDS group was similar to that in the control group (67 ± 22% vs. 70 ± 28%). The numbers of W5 CFC in the MDS cultures were in the same range as those in the control cultures (Fig. 11).

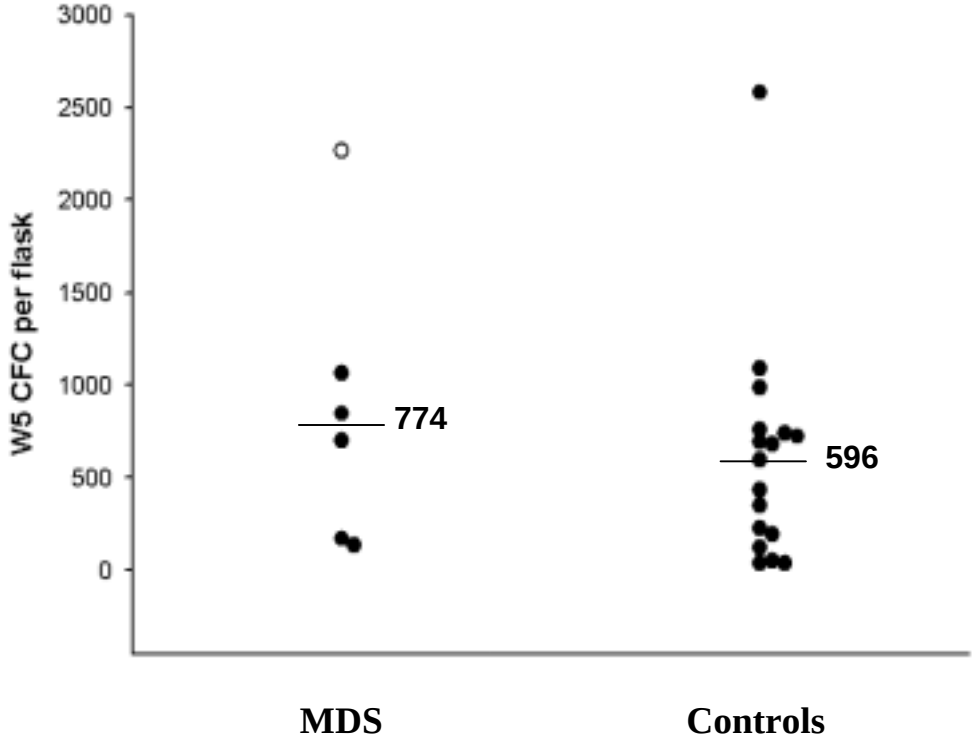

Fig. 11: Numbers of week 5 colony-forming cells (W5 CFC) in two-stage long-term bone marrow cultures from 6 MDS patients and 17 controls. Five MDS patients had prognostically favorable subtypes of the disease and one (open circle) had refractory anemia with excess of blasts (cf. Table 6). After reaching confluency, the cultures were irradiated with 15 Gy and overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

3.2.2 Hematopoiesis-supporting function of stroma fibroblasts

Fibroblasts were purified from confluent stroma of long-term cultures, recultured until confluency, and then overlaid with CD34+ indicator cells. Five of six fibroblast cultures reached confluency while the fibroblasts layer from one patient (no. 2 [TG], cf. Table 6) was too sparse to be able to serve as a support layer for CD34+ cells. Although the range of W5 CFC numbers from MDS and control cultures showed some overlap, a statistically significant impairment of the hematopoiesis-supporting function was seen in the group of MDS cultures ($P = 0.038$; Fig. 12).

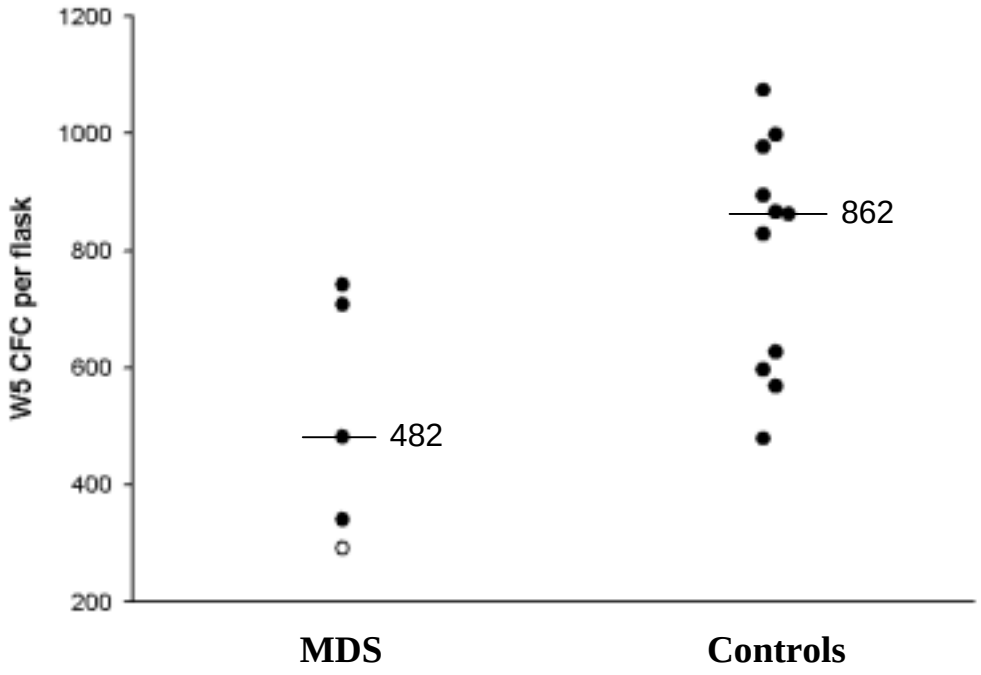

Fig. 12: Numbers of week 5 colony-forming cells (W5 CFC) in pure stroma fibroblast cultures from 5 MDS patients and 11 controls. Four MDS patients had prognostically favorable subtypes of the disease and one (open circle) had refractory anemia with excess of blasts (cf. Table 6). After reaching confluency, the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

3.2.3 Hematopoiesis-supporting function of stroma macrophages

Purified stroma macrophages were co-cultured with M2-10B4 cells as described for the group of AML patients, and evaluated for their ability to support the development of W5 CFC from added CD34+ cells. Figure 13 summarizes the results for those 5 MDS cultures that were prepared using early passage M2-10B4 cells. There were no significant differences between MDS cultures and controls. The results of the only patient (no. 4 [PM]) studied with late passage M2-10B4 cells are not shown.

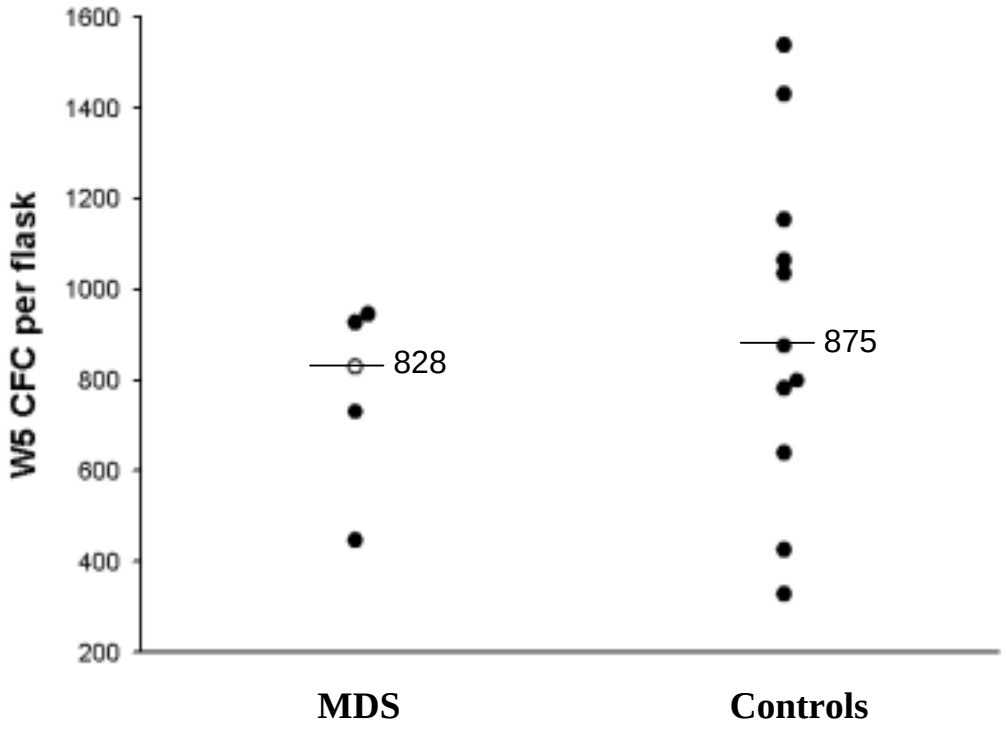

Fig. 13: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of 'early passage' M2-10B4 cells with stroma macrophages from 5 MDS patients and 11 controls. Four MDS patients had prognostically favorable subtypes of the disease and one (open circle) had refractory anemia with excess of blasts (cf. Table 6). The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

3.3 Stroma function in multiple myeloma

3.3.1 Hematopoiesis-supporting function of unfractionated bone marrow stroma

To evaluate the hematopoiesis-supporting function of the entire bone marrow stroma, primary long-term cultures from bone marrow aspirates from 9 multiple myeloma patients were grown to confluency, irradiated and reseeded with purified CD34+ cells as described for the AML group. The control cultures were the same as those used in the AML and MDS studies. The average confluency of the adherent layer in the multiple myeloma group was similar to that in the control group (67 ± 34% vs. 70 ± 28%). The numbers of W5 CFC in the multiple myeloma cultures were in the same range as those in the control cultures (Fig. 14).

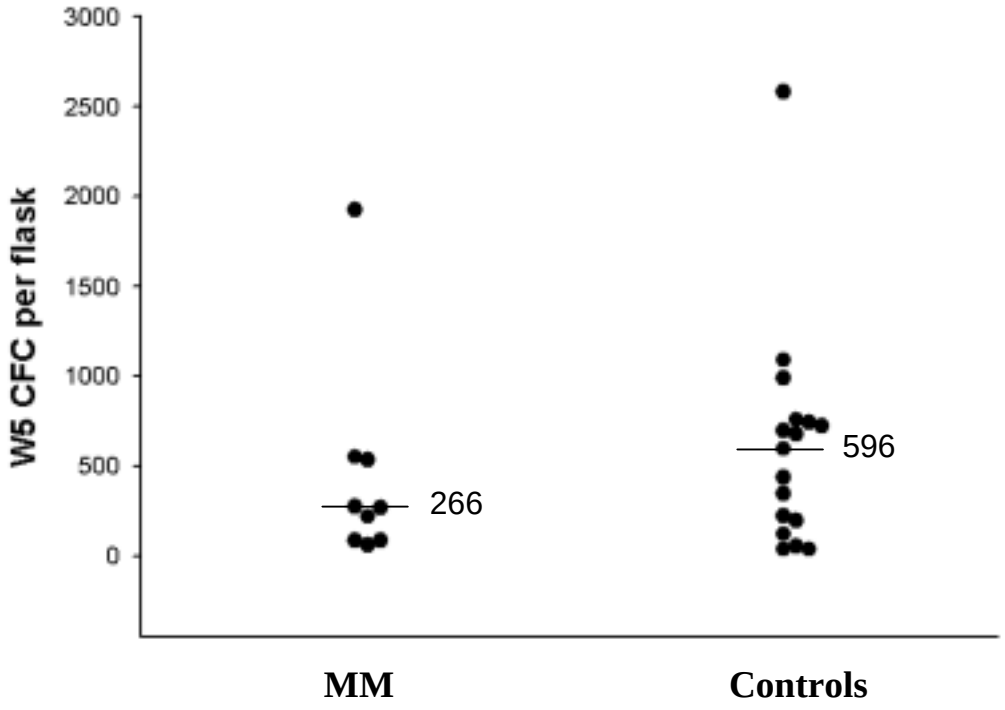

Fig. 14: Numbers of week 5 colony-forming cells (W5 CFC) in two-stage long-term bone marrow cultures from 9 multiple myeloma (MM) patients and 17 controls. After reaching confluency, the cultures were irradiated with 15 Gy and overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

3.3.2 Hematopoiesis-supporting function of stroma fibroblasts

Fibroblasts were purified from confluent stroma of long-term cultures from 7 multiple myeloma patients, recultured until confluency, and then overlaid with CD34+ indicator cells. In two patients (no. 3 [PL] and no. 9 [SK], cf. Table 7) the cellularity of the marrow aspirate was insufficient to allow the establishment of long-term cultures for fibroblast purification. Although the range of W5 CFC values in the group of MM cultures appeared to be somewhat wider than in the controls, a statistically significant difference between the two groups was not observed (Fig. 15).

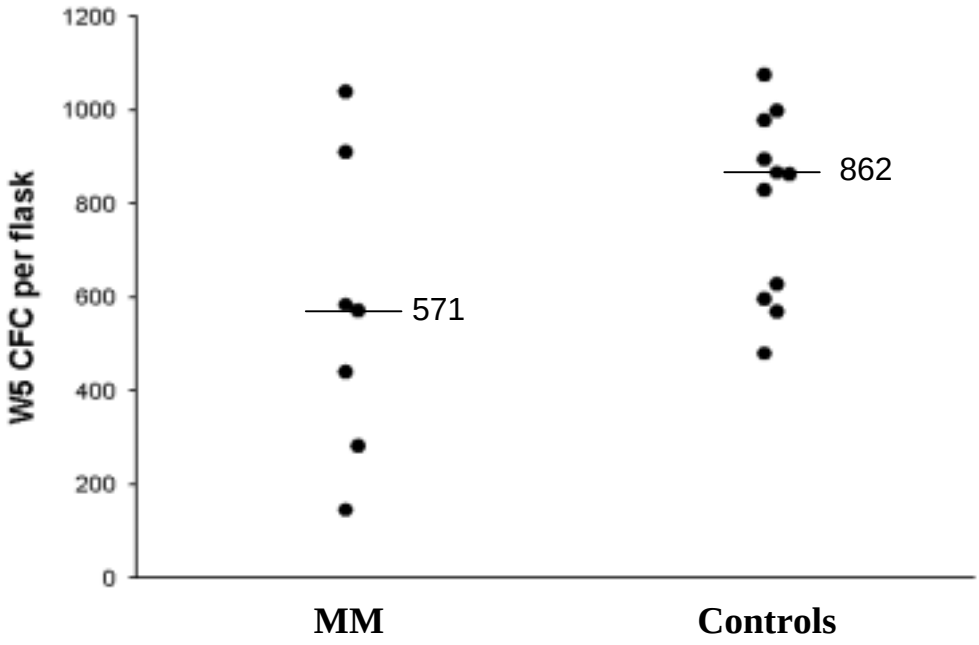

Fig. 15: Numbers of week 5 colony-forming cells (W5 CFC) in pure stroma fibroblast cultures from 7 multiple myeloma (MM) patients and 11 controls. After reaching confluency, the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

3.3.3 Hematopoiesis-supporting function of stroma macrophages

Purified stroma macrophages from 8 multiple myeloma patients were co-cultured with M2-10B4 cells as described for the group of AML patients, and evaluated for their ability to support the development of W5 CFC from added CD34+ cells. In one patients (no. 5 [KC], cf. Table 7) the cellularity of the marrow aspirate was insufficient to allow the establishment of long-term cultures for macrophage purification. Similar to the investigation in AML patients, two different batches of M2-10B4 cells of differing hematopoiesis-supporting potential were used in these experiments. Figure 16a summarizes the results for the cultures prepared with late passage M2-10B4 cells, and Figure 16b shows the results obtained with early passage M2-10B4 cells. In neither of the two sets of experiments were significant differences observed between multiple myeloma and control cultures. The ratio between the median W5 CFC numbers observed in the set of experiments using early passage M2-10B4 cells and the set of experiments using late passage M2-10B4 cells was similar in the groups of multiple myeloma patients (7.72) and controls (8.93). The results of the two sets of experiments were therefore combined by mathematical recalculation of the data, as described in the AML section. Again, there were no significant differences between multiple myeloma cultures and controls (Fig. 16c).

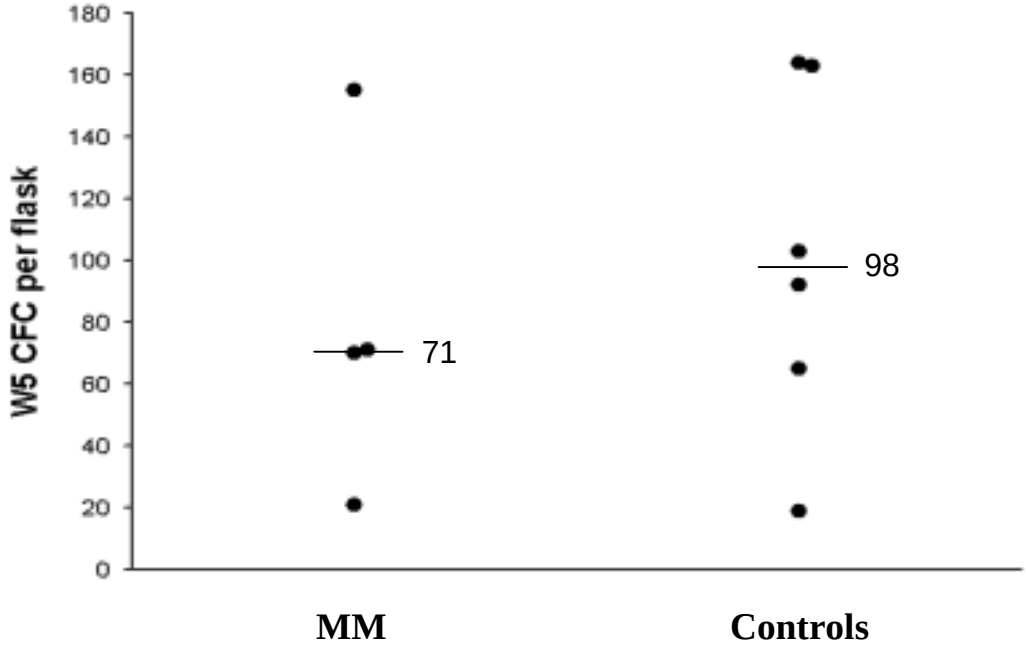

Fig. 16a: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of 'late passage' M2-10B4 cells with stroma macrophages from 4 multiple myeloma (MM) patients and 6 controls. The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

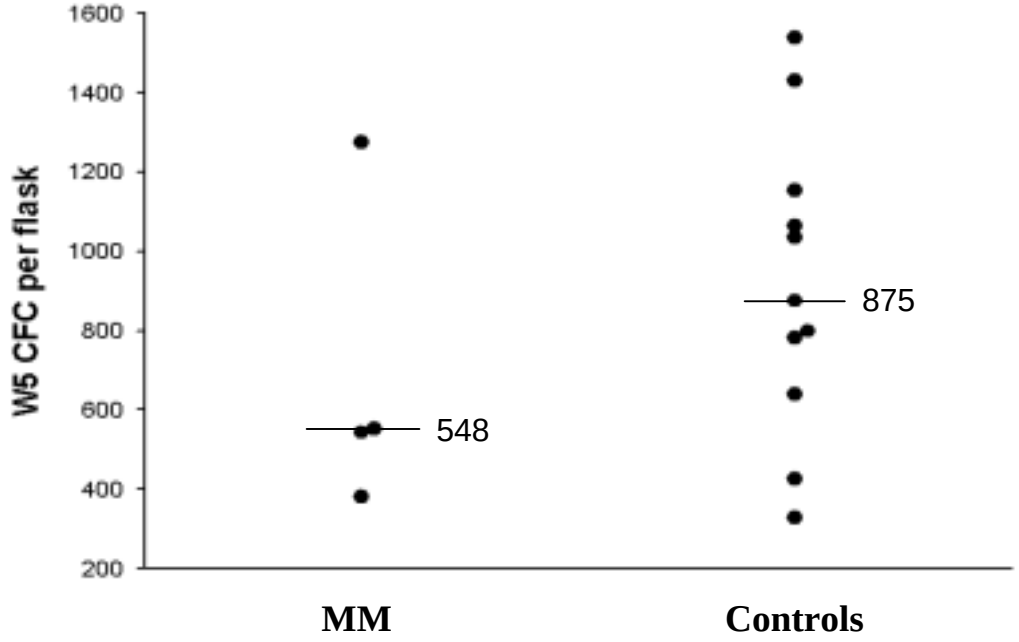

Fig. 16b: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of 'early passage' M2-10B4 cells with stroma macrophages from 4 multiple myeloma (MM) patients and 11 controls. The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. Lines represent median values.

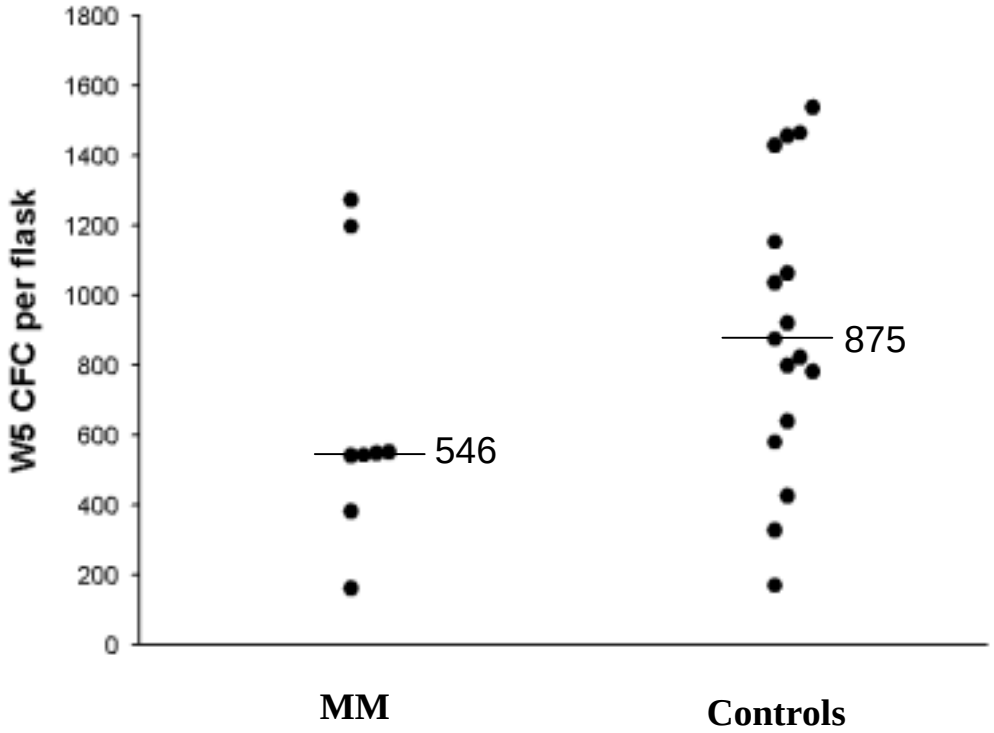

Fig. 16c: Numbers of week 5 colony-forming cells (W5 CFC) in co-cultures of 'early' or 'late passage' M2-10B4 cells with purified stroma macrophages from 8 multiple myeloma (MM) patients and 17 controls. The macrophages were added to irradiated confluent M2-10B4 layers, and the cultures were overlaid with 2.5×10^4 CD34+ cells. The data generated with cultures containing 'late passage' M2-10B4 cells were recalculated as described in the text. Lines represent median values.

3.4 Differences in the hematopoiesis-supporting function of early versus late passage M2-10B4 cells

As mentioned in section 3.1.2.4, a fresh batch of M2-10B4 cells was thawed in the course of the experiments because the originally used cells grew poorly and provided weak support for co-cultured CD34+ cells. The new batch termed 'early passage' M2-10B4 cells was about 9-times more potent in supporting blood cell formation in conjunction with human macrophages than the original batch referred to as 'late passage' cells. Interestingly, the two batches of M2-10B4 cells did not only differ quantitatively with respect to their ability to support hematopoesis, but they also differentially affected the behavior of co-cultured macrophages and the ratio between precursor cells and non-adherent end cells in the cultures which reflects the balance between stem cell maintenance and differentiation.

The differential effect of the two batches of M2-10B4 cells on co-cultured macrophages became apparent when the hematopoiesis-supporting capacity of M2-10B4 cultures supplemented with macrophages were compared with that of control cultures containing M2-10B4 cells alone (Figs. 17a and b). In M2-10B4 control cultures the median number of W5 CFC per flask was 174 for late passage and 306 for early passage M2-10B4 cells. Figure 17 depicts the ratio between W5 CFC numbers in macrophage-supplemented cultures and their respective M2-10B4 only controls. A ratio below 1 signifies a detrimental effect of macrophages on M2-10B4-mediated stem cell maintenance, while a ratio above 1 means that macrophages enhance the stem cell-supporting effect of M2-10B4 cells. With late passage M2-10B4 cells, an inhibitory effect of co-cultured macrophages on stem cell maintenance was observed in the majority of cultures (Fig. 17a). By contrast, when early passage M2-10B4 cells were used as a feeder layer, the addition of macrophages almost invariably led to improved stem cell maintenance. As described in sections 3.1.2.4, 3.2.3 and 3.3.3, stroma macrophages from AML patients had a significantly stronger potency to support the development of W5 CFC than macrophages from controls (Figs. 8a-c) or patients with MDS (Fig. 13) or multiple myeloma (Figs.16a-c).

The number of non-adherent cells in the supernatant reflects the productivity of the long-term culture which is a function of the number of stem cells in the stroma layer and the number of mature cells produced by each stem cell. In M2-10B4 control cultures the median number of non-adherent cells per flask at week 5 was 0.20×10^5 for late passage and 0.80×10^5 for early

passage M2-10B4 cells. Figures 18a and b show that the addition of human macrophages to the adherent layer increased the production of mature cells irrespective of the batch of M2-10B4 cells used.

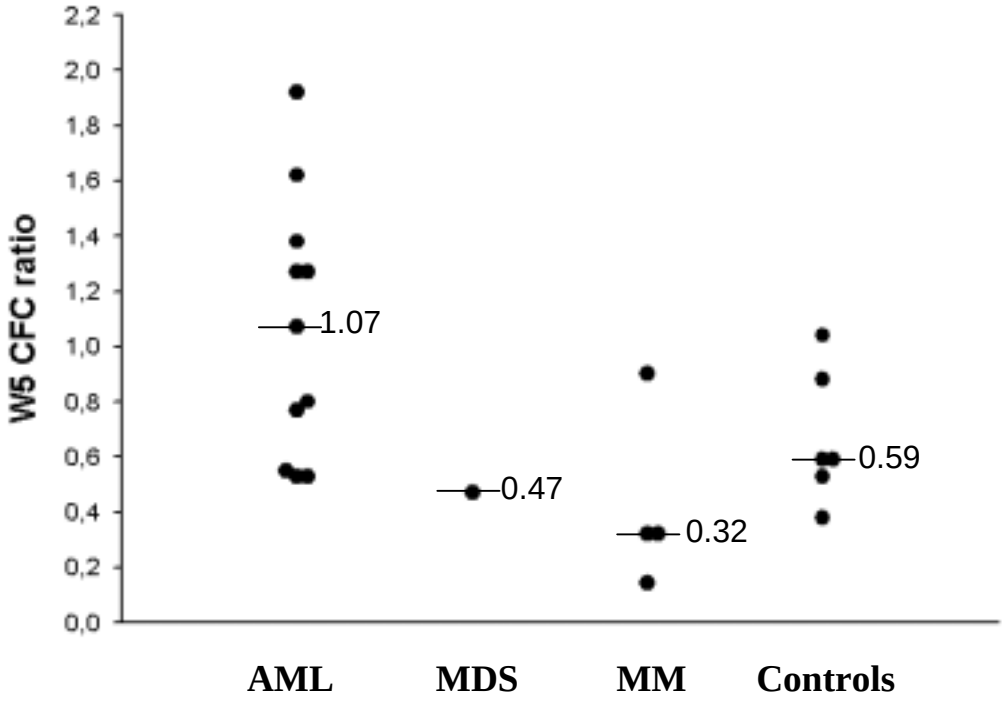

Fig. 17a: Ratio of W5 CFC in 'late passage' M2-10B4 cultures supplemented with $2.5x10^4$ CD34+ cells plus $3x10^5$ macrophages and M2-10B4 cultures supplemented with CD34+ cells alone. The median absolute W5 CFC number in M2-10B4 cultures supplemented with CD34+ cells alone was 174 per flask (range 50-271).

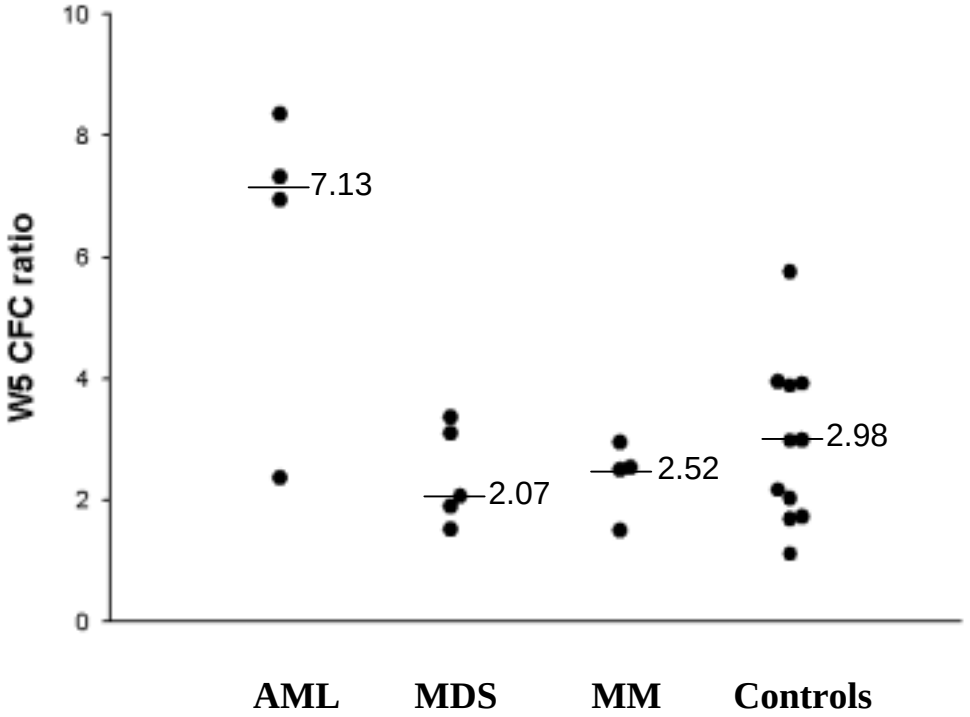

Fig. 17b: Ratio of W5 CFC in 'early passage' M2-10B4 cultures supplemented with $2.5x10^4$ CD34+ cells plus $3x10^5$ macrophages and M2-10B4 cultures supplemented with CD34+ cells alone. The median absolute W5 CFC number in M2-10B4 cultures supplemented with CD34+ cells alone was 306 per flask (range 254-889).

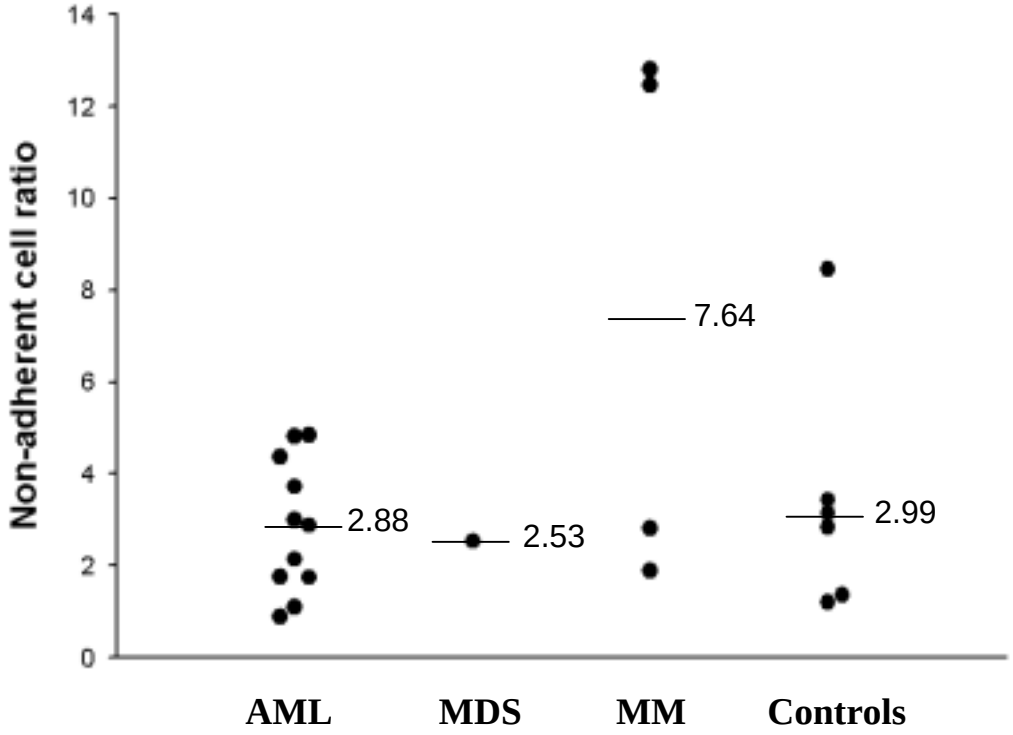

Fig. 18a: Ratio of total non-adherent cells at week 5 in 'late passage' M2-10B4 cultures supplemented with $2.5x10^4$ CD34+ cells plus $3x10^5$ macrophages and M2-10B4 cultures supplemented with CD34+ cells alone. The median absolute number of non-adherent cells in M2-10B4 cultures supplemented with CD34+ cells alone was $0.20x10^5$ per flask (range $0.06\text{-}0.45x10^5$).

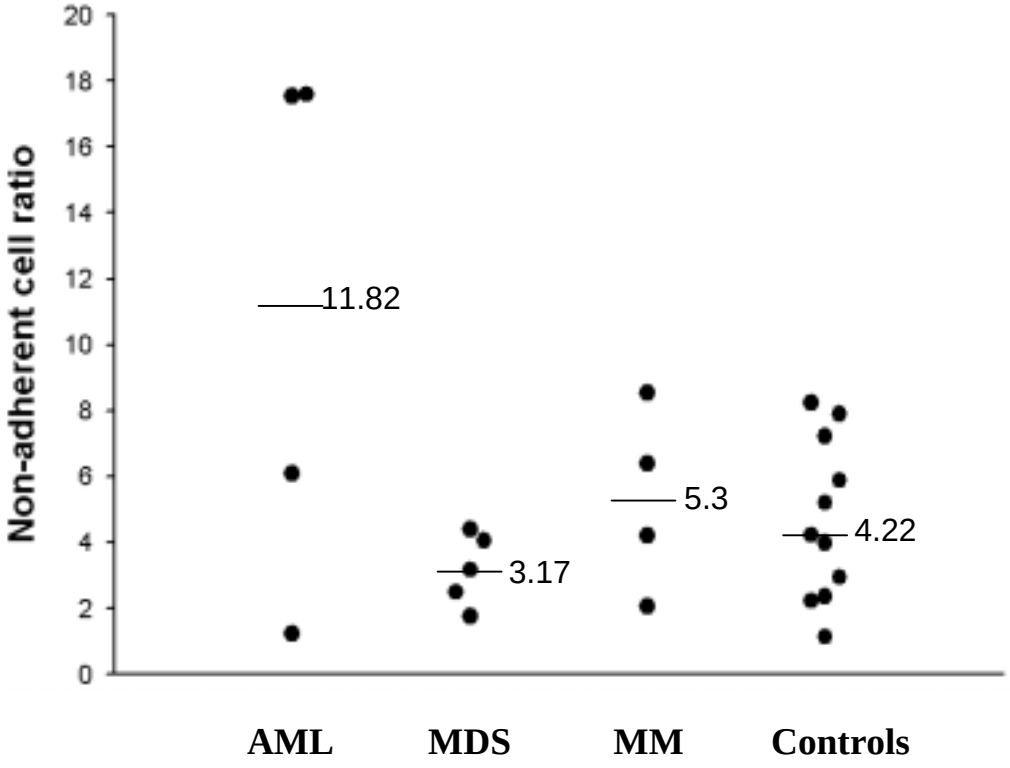

Fig. 18b: Ratio of total non-adherent cells at week 5 in 'early passage' M2-10B4 cultures supplemented with $2.5x10^4$ CD34+ cells plus $3x10^5$ macrophages and M2-10B4 cultures supplemented with CD34+ cells alone. The median absolute number of non-adherent cells in M2-10B4 cultures supplemented with CD34+ cells alone was $0.80x10^5$ per flask (range $0.02\text{-}1.89x10^5$).

The relation between the number of total non-adherent cells in the supernatant at week 5 and the number of W5 CFC indicates the balance between stem cell differentiation and maintenance. When early passage M2-10B4 cells which provide favorable conditions for stem cell maintenance were used as a feeder layer, macrophages did not significantly perturb the ratio between stem cell maintenance and differentiation (Fig. 19). By contrast, in poor culture conditions provided by late passage M2-10B4 cells, a striking difference was observed between the function of macrophages from AML patients and macrophages from controls or patients with MDS or multiple myeloma. While the latter appeared to promote differentiation, AML macrophages seemed to favor maintenance of stem cells (Fig. 19).

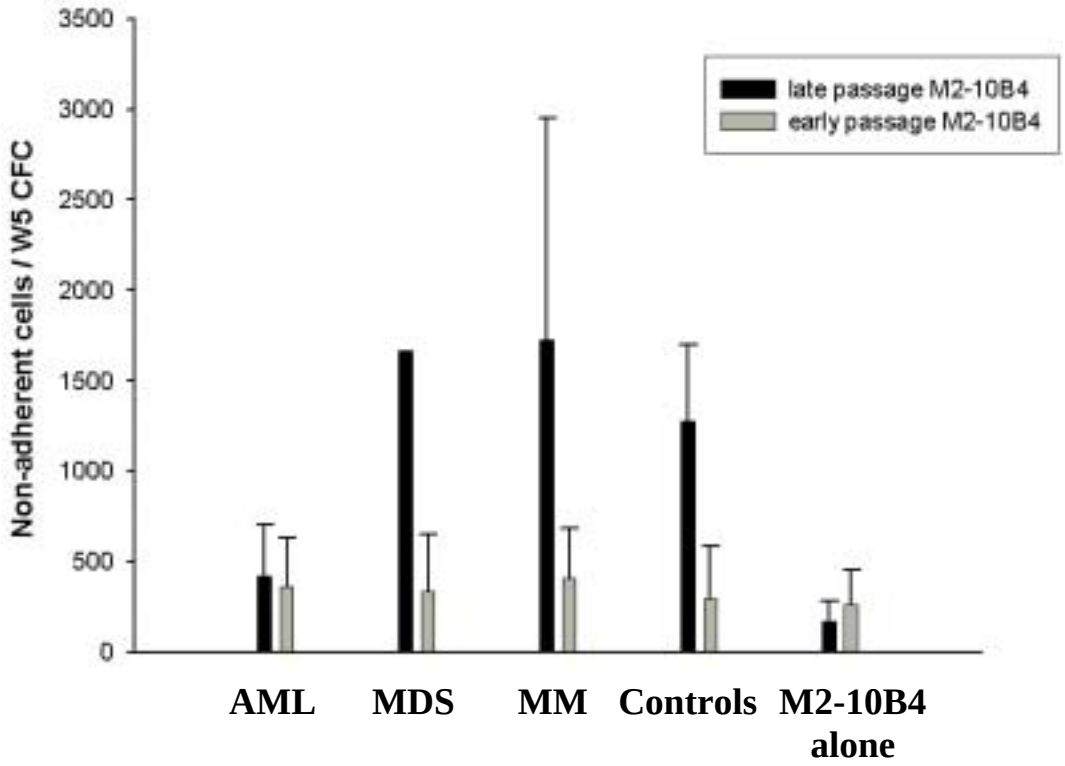

Fig. 19: Ratio between total non-adherent cells at week 5 and W5 CFC in 'early' or 'late passage' M2-10B4 cultures supplemented with $2.5x10^4$ CD34+ cells plus $3x10^5$ macrophages (designated AML, MDS, MM or controls, respectively) or CD34+ cells without macrophages (designated M2-10B4 alone). Means ± standard deviation. Note the difference in the behavior of AML macrophages and all other types of macrophages in cultures prepared with 'late passage' M2-10B4 (P = 0.034).

4 Discussion

The long-term bone marrow culture system is regarded as the best in vitro model for hematopoiesis. In this culture system, hematopoietic stem cells reside in the stromal microenvironment where they proliferate and differentiate. Maturing progeny and post-mitotic end cells are then released into the culture medium. A well-balanced interaction between hematopoietic cells and stroma cells is a prerequisite to sustain long-term hematopoiesis. Maintenance of hematopoiesis for prolonged periods of time does not only depend on the quantitative and qualitative state of hematopoietic stem cells, but also on the function of the stromal microenvironment.

One of the major drawbacks of the long-term culture system is the fact that, even if the experimental conditions are strictly standardized and homogeneous populations of cells from healthy individuals or patients are analysed, the results may be quite variable. The reason for this variability which may have prevented a more wide-spread use of the system in experimental hematology, is not entirely understood. Although a high degree of heterogeneity was also observed in the study presented here, the results demonstrate clear differences in the behavior of stroma fibroblasts and/or macrophages from patients with myeloid malignancies as compared to the respective cells from individuals with a healthy bone marrow or multiple myeloma.

4.1 Function of bone marrow stroma cells in acute myeloid leukemia

In primary long-term cultures of AML bone marrow, two patterns of progenitor cell growth were observed highlighting the heterogeneity of the disease. These findings are in line with those reported by Coulombel et al. (Coulombel et al., 1985). When cultures with an obvious overgrowth of leukemic cells were excluded from the analysis, a more homogeneous pattern with decreased numbers of progenitor cells was observed, implying that normal hematopoiesis is suppressed in long-term cultures from AML bone marrow. Three possible reasons may contribute to this result: First, although the same numbers of mononuclear cells were seeded initially in AML and control cultures, a preponderance of leukemic blasts in the AML cultures may dilute the frequency of stem cells with the potential to maintain long-term blood cell formation. Second, leukemic cells have been reported to produce inhibitory factors

interfering with the proliferation of normal hematopoietic cells. And third, abnormalities in the stromal microenvironment may exist in AML. Our investigation focussed on the last named mechanism. Using stroma layers depleted of hematopoietic and leukemic cells by irradiation or immunomagnetic cell separation and reseeded with a standardized inoculum of normal CD34+ cells, we were able to analyse the hematopoiesis-supporting function of unfractionated stroma or purified stroma cell components without the confounding effects of co-cultured leukemic cells.

The function of the entire unfractionated bone marrow microenvironment was analysed by employing the two-stage culture model with irradiated stroma layers. Using this assay system we were unable to detect significant differences between AML stroma and stroma from healthy bone marrow. In contrast to our results, other reports hint at an altered function of long-term culture stroma from AML patients. Mayani et al. used normal bone marrow buffy coat cells depleted of adherent cells as indicator cells for the hematopoiesis-supporting function of AML stroma and observed a decreased W5 CFC output compared to normal stroma (Mayani et al., 1992a). The decrease in hematopoiesis-supporting capacity correlated with increased levels of TNF-α in the culture medium which was thought to be the main inhibitory factor. By contrast, Dührsen et al. used cells of the transplantable murine leukemia PGM-1 as indicator cells for stroma function and observed a heightened PGM-1-supporting ability of AML stroma as compared to normal stroma (Dührsen et al., 1995). The picture emerging from this discrepancy is that AML stroma may stimulate the production of leukemic cells at the expense of normal blood cells. It cannot be ruled out, however, that differences in the patient population studied or the culture conditions chosen also contributed to the disparity of the results.

The hematopoietic microenvironment is composed of several types of cells which are involved in the regulation of hematopoiesis by direct cell-cell contact, production of cytokines or elaboration of an extracellular matrix. Every cell component contributes to the global function of the entire stroma. In order to clarify whether the seemingly unperturbed function of the unfractionated stroma layer in AML was due to an unperturbed function of its individual cellular constituents or to mutual compensation of functional alterations of different stroma components, the main cell types of the stroma, fibroblasts and macrophages, were purified from the adherent layer and analysed separately.

In contrast to the function of the intact stroma layer, the hematopoiesis-supporting ability of purified fibroblasts was shown to be decreased in the AML group. The decrease was not due to insufficient fibroblast numbers because, in all but one of the AML patients, the isolated fibroblasts could form a confluent adherent layer. Our result is in keeping with the report by Greenberg et al. who also demonstrated a deficient ability of the fibroblast layers from AML patients to support hematopoiesis in vitro (Greenberg et al., 1981). The functional deficiency has been shown to be associated with an abnormal expression of cytokines including IL-1β, IL-6, G-CSF and leukemia inhibitory factor (LIF) (Denkers et al., 1992; Wetzler et al., 1995) and a reduced membrane expression of certain adhesion molecules (Denkers et al., 1992; Wetzler et al., 1995). The abnormal function may be orchestrated by coexisting leukemic cells. Inhibitory effects on fibroblast precursors by AML cells, AML cell-conditioned medium, and AML serum have been reported (Nagao et al., 1983b; Nara et al., 1984). In the experiments presented here, however, significant numbers of contaminating AML cells were not present in the population of purified fibroblasts. Therefore, a more likely explanation for the functional abnormalities is the assumption that the establishment of an AML cell population in the bone marrow leads to a shift in the relative frequency of different types of stroma fibroblasts which results in a decreased hematopoiesis-supporting ability in the long-term culture system. This assumption is supported by evidence from other investigators (Dührsen and Hossfeld, 1996; Greenberg et al., 1984)**.**

Isolated stroma macrophages are unable to form a confluent adherent layer and maintain long-term hematopoiesis. We therefore studied their function in the control of blood cell formation by standardized co-culture with a confluent layer of irradiated M2-10B4 cells. M2-10B4 is a murine bone marrow fibroblast cell line which, despite its inability to secrete human hematopoietic growth factors, can sustain human long-term hematopoiesis as effectively as human fibroblasts (Sutherland et al., 1991). Its use as a feeder layer satisfied the following requirements:

1. Preliminary experiments showed that the presence of minute numbers of contaminating fibroblasts within the population of purified macrophages resulted in rapid overgrowth of this highly proliferating cell type. Since the growth and survival of fibroblasts in vitro is dependent on adherence to plastic, we reasoned that a pre-established layer of M2-10B4 cells would deprive the fibroblasts of anchor sites on the bottom of the culture flask thus preventing their proliferation.

2. Preliminary experiments also showed that a substantial proportion of purified macrophages died after transfer to a new flask and subsequent culture at low cell density. Transfer of the cells to a flask with a pre-established layer of M2-10B4 cells resulted in substantially improved macrophage survival.
3. The type of feeder layer used for the study of macrophage function had to be kept constant throughout the investigation. Since M2-10B4 is a continuous cell line, there were always sufficient cells for the experiments.

To our knowledge, our approach to study the function of isolated stroma macrophages in co-culture with a standard adherent layer, has not been taken before. Although a considerable amount of heterogeneity was observed, the assay system permitted the detection of a significantly heightened hematopoiesis-supporting function of macrophages from AML patients as compared to the cells from controls or patients with myelodysplastic syndromes or multiple myeloma.

Our findings are somewhat similar to those of Mayani et al. who observed an increased W5 CFC output in two-stage long-term cultures from AML bone marrow when macrophage colony-stimulating factor was added during the development of the adherent layer (Mayani et al., 1992c). This cytokine induced the growth of increased numbers of macrophages which were supposed to be of leukemic origin. The authors interpreted their observation as a reduced capacity of leukemic (as opposed to normal) macrophages to produce the hematopoietic inhibitor TNF-α (Mayani et al., 1992c). These findings contrast with those of Greenberg et al. who used monocyte-macrophage populations from AML patients and controls to stimulate the growth of progenitor cells in agar cultures (Greenberg et al., 1978). Cells of AML origin provided a lower capacity to promote granulocyte colony formation than cells from controls, with an improvement in macrophage function upon achievement of disease remission. The discrepancy between the results reported by Mayani et al. and Greenberg et al. was probably related to the fact that their assay systems were not identical.

Since stroma macrophages are of hematopoietic origin, it is conceivable that, in patients with AML, they are part of the leukemic clone. A leukemic origin of marrow macrophages has been demonstrated in several instances of acute myeloid leukemia (Langley et al., 1986; Zhang et al., 1999). A systematic study using cytogenetic markers, however, revealed that, in most cases of AML, the majority of stroma macrophages is not derived from the leukemic clone (Zhang et al., 1999).

The simplest explanation for our findings is the assumption that, in patients with AML, a decrease in the hematopoiesis-supporting function of stroma fibroblasts was compensated for by an increase in the hematopoiesis-supporting function of stroma macrophages. Overall, the hematopoiesis-supporting ability of the unfractionated stroma layer was not significantly different from that of patients with a healthy bone marrow. Our results show that, even if no abnormal function is detected in the unfractionated stroma layer, an abnormality may still exist in specific stroma components. These may exert unusual regulatory effects possibly promoting the growth of leukemic and inhibiting the growth of normal hematopoietic cells (Dührsen and Hossfeld, 1996).

In 5 patients with AML, the function of the bone marrow stroma was analysed both before and after administration of chemotherapy. All patients experienced a decrease in the hematopoiesis-supporting function of unfractionated stroma and purified stroma macrophages, and all but one also showed a decrease in the function of purified stroma fibroblasts after chemotherapy. Interestingly, the patient whose fibroblast function improved following chemotherapy was the only one to attain complete remission. While the small number of patients included in this part of the study precludes a meaningful interpretation of the results, disease stage-dependent fluctuations in stroma function or composition have also been reported by other investigators (Greenberg et al., 1981; Kaneko et al., 1982; Katsuno et al., 1986; Nagao et al., 1983a). The numbers of fibroblast precursors (colony-forming unit-fibroblast, CFU-F) and the support function of fibroblast layers were decreased at initial diagnosis and relapse, and recovered to almost normal levels at remission. Although chemotherapy may improve the function of the hematopoietic microenvironment by eliminating neoplastic cells, its predominant effect is deterioration of stroma function which is probably related to its cytotoxic properties. This has most convincingly been demonstrated in patients receiving myeloablative therapy prior to autologous or allogeneic stem cell transplantation (Galotto et al., 1999).

4.2 Function of bone marrow stroma cells in myelodysplastic syndromes

The term ‘myelodysplastic syndrome’ encompasses a heterogeneous group of hematopoietic malignancies which is subdivided on morphological grounds according to the French-American-British classification (Bennett et al., 1982). The biological behavior - as evidenced

by the response to therapy, the duration of survival or the rate of transformation to acute leukemia - varies greatly among different subtypes. Not surprisingly, the results of experimental studies on the biology of MDS are characterized by a substantial degree of heterogeneity.

Similar to the findings in AML, considerable variability was also observed in the behavior of long-term cultures from patients with MDS. No statistically significant abnormalities were detected when unfractionated stroma or isolated stroma macrophages were analysed. With regard to unfractionated stroma our findings are confirmed by several reports from other researchers (Coutinho et al., 1990; Milner et al., 1977; Ruutu et al., 1984). The lack of evidence for abnormalities in the hematopoiesis-supporting function of MDS stroma was paralleled by an inability to detect abnormalities in its cytokine expression pattern (Hirayama et al., 1993). Other authors, however, reported a decreased hematopoiesis-supporting function of bone marrow stroma from MDS patients (Aizawa et al., 1999; Tennant et al., 2000). This controversy is most likely related to differences in patient populations studied and experimental conditions chosen. The same may be true for a report of an aberrant macrophage function in MDS patients characterized by increased production of inhibitory cytokines upon stimulation with GM-CSF (Ohmori et al., 1990).

The only abnormality to be detected in our analysis in MDS patients was a decreased hematopoiesis-supporting ability of purified stroma fibroblasts. The most dramatic decrease was observed in a patient with refractory anemia with excess of blasts, a subtype of MDS with a very high likelihood to proceed to acute myeloid leukemia. Abnormalities in the behavior of stroma fibroblasts from MDS patients have also been reported by other investigators. Several groups reported a reduced frequency of CFU-F or an incomplete confluency of the adherent layer in long-term bone marrow cultures indicative of a defect in cell survival or proliferation (Borbenyi et al., 1987; Coutinho et al., 1990; Zipori et al., 1985). Failure to develop a confluent adherent layer was also observed in a culture of purified fibroblasts from one of our patients. The reduced hematopoiesis-supporting function of MDS fibroblasts may be related to the ability of stroma cells from some MDS patients to induce apoptosis in hematopoietic cells (Aizawa et al., 1999; Aizawa et al., 2000; Tauro et al., 2002). Furthermore, stroma cells from MDS patients may show abnormal cytokine expression, including an increased expression of IL-1β, TGF-β, IL-6 and LIF and a decreased expression of IL-1α receptor antagonist (Raza et al., 1995a; Raza et al., 1995b; Wetzler et al., 1995). There may also be

abnormal adhesive interactions and abnormalities in the composition of the extracellular matrix which is, to a large extent, produced by stroma fibroblasts. The prognostically unfavorable occurrence of 'atypically located immature precursors' (ALIPs) which is reported in about 60% of MDS cases may be related to such abnormal interactions between hematopoietic cells and stroma fibroblasts (Tricot et al., 1984).

4.3 Function of bone marrow stroma cells in multiple myeloma

To keep the results comparable with the AML and MDS groups, we also used Dexter-type long-term cultures (Dexter et al., 1977) to study stroma function in multiple myeloma patients. Multiple myeloma is a human B-cell neoplasm. The use of Dexter-type cultures which is conducive to the establishment of myeloid hematopoiesis may prevent the detection of abnormalities related to lymphoid hematopoiesis. Therefore, our results do not exclude the possibility that culture conditions promoting the growth of lymphoid cells (Whitlock and Witte, 1982) would have led to the development of an adherent layer with different properties.

Irrespective of the composition of the stromal support layer (unfractionated stroma, purified fibroblasts, purified macrophages co-cultured with M2-10B4 cells), differences between multiple myeloma cultures and cultures established from healthy bone marrow were not observed. Subtle differences in fibroblast function described by others (Gregoretti et al., 1994), such as a reduced deposition of extracellular matrix proteins, remained undetectable in our assay system.

4.4 Fibroblast heterogeneity as demonstrated by differing functional properties of early versus late passage M2-10B4 cells

An unexpected side finding of our study was the observation that M2-10B4 cells of dissimilar age differed in their capacity to support hematopoiesis. In addition, the batch of M2-10B4 cells used had a strong influence on the behavior of co-cultured human macrophages and the relation between the numbers of immature precursors (W5 CFC) and mature end cells at the end of the 5-week culture period.

When late passage M2-10B4 cells were used in conjunction with normal macrophages, the relation between W5 CFC and mature cells was shifted towards the latter, indicating limited self-renewal and preferential differentiation of stem cells. By contrast, with early passage M2-10B4 cells co-cultured with normal macrophages, the ratio was shifted towards W5 CFC. In these cultures there were also very high cell numbers in the supernatant indicating both increased stem cell maintenance and enhanced proliferation of differentiated cells. A model explaining these results is shown in Figure 20.

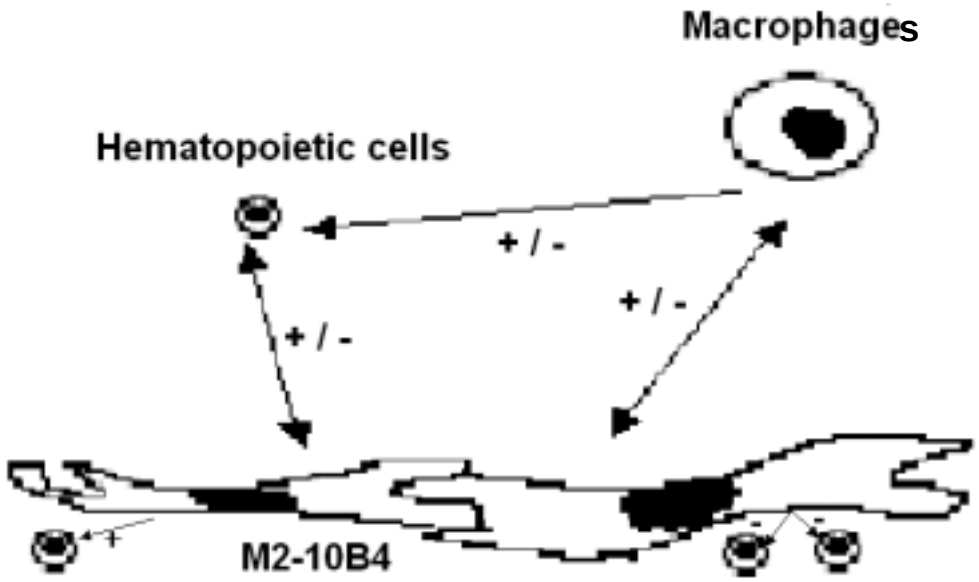

Figure 20: Interactions between M2-10B4 stroma fibroblasts, human stroma macrophages and hematopoietic cells. "+" indicates stimulatory signals which promote differentiation; "-" indicates inhibitory signals which inhibit differentiation but sustain the stem cell pool.

Hematopoietic stem cells are supposed to be located in specific stem cell niches composed of stroma cells and their products. In long-term bone marrow cultures stem cells remain in a quiescent state only to enter the cell cycle upon change of the culture medium (Cashman et al., 1999). If daughter cells associate with another stem cell niche, they also stay in a non-cycling state thus maintaining the stem cell pool. If they are exposed to a part of the microenvironment which does not contain a stem cell niche, they differentiate, proliferate and finally migrate into the culture medium (Verfaillie, 1998). Late passage M2-10B4 cells may have an impaired ability to provide sufficient niches for stem cells, so stem cells will undergo differentiation and maturation under the influence of soluble factors provided by co-cultured human macrophages. The result of such culture conditions is decreased stem cell maintenance with an increased proportion of mature cells in the supernatant. By contrast, early passage M2-10B4 cells appear to be able to provide enough niches for dividing stem cells thus maintaining and expanding the pool of early precursors. When their daughter cells are hit by

mediators from human macrophages they proliferate and differentiate to more mature cells which are eventually released into the supernatant. As a result, both increased stem cell maintenance and increased production of differentiated progeny are demonstrable in these cultures.

In contrast to macrophages from healthy bone marrow, macrophages from AML marrow tended to promote maintenance rather than differentiation of stem cells. Whether the stimulation of self-renewal extends to leukemic stem cells which could contribute to an amplification of the leukemic clone, remains to be demonstrated.

4.5 Conclusions and outlook

Using the two-stage long-term culture system as an instrument to analyse stroma function without the confounding effects of benign or malignant hematopoietic cells we were able to demonstrate a decreased hematopoiesis-supporting function of fibroblasts from patients with AML and MDS and an increased hematopoiesis-supporting function of macrophages from patients with AML in comparison to the respective stroma cell types from individuals with a healthy bone marrow or multiple myeloma. Although these differences reached statistical significance, the variability observed within the groups of patients with AML or MDS suggests that some patients harbor stroma cells with abnormal properties whereas, in others, cell function may still be normal. The major questions arising from these results concern the clonal origin of the abnormal cells and the molecular basis of their perturbed function. During the course of our experiments cytospins were prepared and RNA was extracted from purified stroma cell populations whenever the volume of the initial bone marrow aspirate permitted such preparations. Our future work will be directed at analysing these samples by interphase fluorescence in situ hybridisation with chromosome probes specific for the cytogenetic abnormalities of the respective diseases and by establishing gene expression profiles using microarray technology. It is hoped that these studies will provide further insight into the abnormalities of the bone marrow microenvironment in hematological malignancies.

5 Summary

The long-term bone marrow culture system is the most frequently used in vitro model for hematopoiesis. In this system, the adherent layer, composed of stroma cells of non-hematopoietic (fibroblasts, adipocytes, endothelial cells) or hematopoietic origin (macrophages) and their products (adhesion molecules, cytokines, extracellular matrix), constitutes the hematopoietic microenvironment which orchestrates the proliferation, differentiation and maturation of hematopoietic stem cells and their progeny.

To investigate stroma function in hematological malignancies, we established long-term bone marrow cultures from 21 untreated patients with acute myeloid leukemia (AML), 6 untreated patients with myelodysplastic syndromes (MDS), 9 untreated patients with multiple myeloma and 28 controls, and measured the hematopoiesis-supporting ability of the stroma by determining the number of colony-forming cells in the adherent layer and the supernatant after 5 weeks' of culture (W5 CFC). In order to study stroma function without the confounding effects of endogenous benign or malignant hematopoietic cells the cultures were either irradiated or stroma fibroblasts and macrophages were purified using immunomagnetic techniques. While stroma fibroblasts could be used directly as a support layer for co-cultured CD34+ indicator cells, purified stroma macrophages were unable to form a confluent adherent layer and had to be analysed in conjunction with irradiated murine M2-10B4 cells.

In contrast to purified fibroblasts from healthy or multiple myeloma bone marrow, fibroblasts from AML and MDS marrow showed a significantly reduced capacity to support blood cell formation. The hematopoiesis-supporting ability of stroma macrophages from AML marrow was markedly enhanced in comparison to macrophages from MDS, multiple myeloma or control marrow. Although the results were statistically significant, considerable overlap was observed in the range of activities of stroma cells from controls and patients, suggesting that the occurrence of functionally abnormal cells was limited to a subfraction of AML and MDS patients. Longitudinal studies showed that chemotherapy of patients was followed by a deterioration of stroma function.

The function of stroma fibroblasts and macrophages is markedly perturbed in a substantial proportion of patients with myeloid malignancies. The major questions arising from these observations concern the clonal origin of the abnormal cells and the molecular basis of their perturbed function.

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