Regulatory Interactions in Normal and Leukemic Myelopoiesis*

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1. Introduction

In vitro culture studies have revealed complex hemopoietic cell interactions involving local and systemic signals controlling cell proliferation and differentiation. Phenotypic characterization of myeloid leukemia utilizing in vitro culture methodologies suggests that subtile regulatory imbalance may play a major role in clonal dominance of transformed pluripotential stem cells or committed granulopoietic progenitor cells. The emerging complexity of potential bioregulatory networks in both normal and neoplastic hemopoiesis has almost exclusively been demonstrated in vitro, however the concept of "dependence" as applied to retention of regulatory responsiveness by neoplastic myelopoietic cells is of sufficient practical and theoretical importance to justify detailed consideration of hemopoietic regulators in the context of human leukemia.

2. Regulatory Networks in Normal and Leukemic Myelopoiesis

I. Control of the granulocyte-macrophage progenitor cell (CFU-c)

Myeloid leukemic cells can be cloned in semi-solid culture medium, in suspension cultures or in diffusion chambers implanted into mice. Confirmation of the leukemic origin of the cells derives from morphology, karyotypic analysis, biophysical characteristics and in vitro clonal growth patterns. As has been extensively documented, normal granulocyte macrophage progenitor cells (CFU-c) exhibit an absolute dependence upon provision of an appropriate source of colony stimulating factor (CSF). Since a major source of CSF resides within the hemopoietic system itself, either from mitogen stimulated lymphocytes or monocytes and macrophages, any consideration of leukemic cell responsiveness to CSF must also include recognition that CSF may itself be a product of leukemic cells. Cells from patients with leukemia can be cloned without addition of exogenous CSF. However, growth is not autonomous and cell separation procedures have demonstrated the presence of endogenous CSF producing cells which could be separated from leukemic colony forming cells [1]. Early studies suggested that CFU-c from patients with myeloid leukemia are heterogeneous in their responsiveness to exogenous

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CSF with acute myeloid leukemic cells generally more responsive than remission or normal CFU-c and chronic myeloid leukemic cells exhibiting decreased sensitivity to stimulation [2]. The question of altered responsiveness of leukemic cells to stimulating factors is, however, complicated by interactions between inhibitory and stimulatory cell populations within leukemic bone marrow and the known functional heterogeneity of CFU-c subpopulations and differing species of CSF.

Recognition that normal and neoplastic monocytes and macrophages can produce CSF and that this factor is an absolute requirement for proliferation and differentiation of normal or neoplastic granulocyte-macrophage progenitor cells introduces the problem of mechanisms designed to counterbalance this positive feedback drive. Limitation of CSF-dependent myelopoiesis may theoretically be mediated by activities inhibiting CSF production by mononuclear phagocytes, by direct inactivation of the CSF molecule or by alteration in the responsiveness of the CFU-c. Evidence for the latter was provided by the observation that the E series prostaglandins (PGE_1, PGE_2) profoundly inhibited normal and leukemic CFU-c proliferation in vitro [3]. This inhibition was prevented by preincubation of bone marrow with the dibenzoxapine hydrazide prostaglandin antagonist SC-19220 providing evidence for a PG receptor on the CFU-c. PGE mediated inhibition was also partially counteracted by increasing the concentration of CSF in the culture system suggesting a dualistic modulation of CFU-c proliferation [3]. The central regulatory role of macrophages in myelopoiesis was further indicated by studies which showed that blood monocytes and tissue macrophages were a major biosynthetic source of prostaglandin E as detected by radioimmunoassay [4] and that this synthesis correlated with the production of a diffusible, dialyzible, non species specific inhibitor of CFU-c proliferation [5]. The apparently paradoxical observation that macrophages produced two activities with mutually antagonistic roles at the CFU-c level can be resolved by three observations.

1. While macrophage activation by agents such as endotoxin or zymosan leads to increased synthesis of CSF and PGE, the former occurs as an acute response peaking within 1–3 hours whereas the latter increase is delayed for 18–24 hours [6].

2. The temporal dissociation between peak induction of CSF and PGE synthesis suggested that PGE biosynthesis may be dependent upon the earlier increase in CSF. This possibility was confirmed by the observation that exposure of macrophages to increasing concentrations of a pure source of CSF led to a subsequent dose dependent increase in PGE synthesis and that this response was also seen with macrophages from endotoxin resistant C3H/HeJ mice [5, 6].

3. By employing sedimentation velocity separation of normal peritoneal cells, it was shown that the cells which produced CSF and synthesized PGE resided within the cell population which was adherent, α-naphthyl esterase positive and phagocytosed latex beads. A subpopulation of these macrophages constitutively produced CSF but little or no PGE ("Helper" macrophages) and were clearly separable from a population of larger macrophages which

constitutively produced PGE and little CSF ("Suppressor" macrophages) [6]. Of particular significance was the observation that the CSF producing – "helper" macrophage population could be induced to extensive PGE production following exposure to endotoxin or an exogenous source of CSF [6].

The relevance of these observations to leukemogenesis resides in the fact that macrophages can function in a helper or suppressor mode to influence myeloid leukemic cell proliferation. Suppression of leukemic cell proliferation by activated macrophages may, for example, be relevant to non-specific host defense against neoplasia. Relatively low numbers ($1-2\times 10^5$/ml) of activated mouse peritoneal macrophages are capable of producing a reversible cytostatic block on a variety of human and murine lymphoid and myeloid leukemic cell lines [7]. This cytostasis was detected by inhibition of leukemic cell growth, ^{3}HTdR incorporation, cloning efficiency and by cytofluorometric analysis. In all cases, cell contact was not required and the diffusible macrophage derived inhibitory activity was prostaglandin E.

While it is often thought that leukemic transformation involves an irreversible maturation block with loss of differentiation function, it is becoming increasingly clear that leukemic cells can, under appropriate conditions, be induced to express various differentiated functions. One example of this is retention by neoplastic myelomonocytic, monocytic and macrophage cell lines of the capacity to constitutively or inducibly synthesize and secrete CSF and prostaglandin E. As can be seen in Table 1, three of the five murine cell lines, the myelomonocytic leukemia WEHI-3, the spontaneous macrophage tumors SK2-2 and PU5.18 and the human adherent cell line SPGcT, constitutively elaborated both CSF and PGE, and like normal macrophages, the augmentation of CSF production following short term incubation with lipopolysaccharide (LPS) was linked to stimulation of PGE synthesis. In contrast to most normal macrophages, the RAW-264 Abelson virus induced macrophage tumor and the Balb/c macrophage cell line J774 did not constitutively produce either CSF or PGE; however, induction of synthesis of both activities followed treatment with LPS. Furthermore, the addition of a source of CSF induced both RAW-264 and J774 to synthesize PGE at levels very similar to those induced by LPS alone. CSF and PGE synthesis by RAW-264 could also be induced by agents other than LPS, such as zymosan, purified protein derivative (PPD) and Poly I-Poly C which also stimulate CSF and PGE production by normal peritoneal macrophages [6]. In this context, treatment of the neoplastic macrophage cell lines with hydrocortisone blocked the induction of CSF and PGE synthesis as well as induction of such functional properties as latex phagocytosis, antibody-dependent phagocytosis of sheep RBC and antibody-dependent lysis of tumor targets; however, hydrocortisone had no effect if these were constitutive properties of the neoplastic cells [8].

II. Granulocyte mediated inhibition of myelopoiesis

The concept of granulocyte mediated negative feedback regulation of myelopoiesis has received some experimental support, however this remains a contentious area due to difficulties in demonstrating in vivo correlates of in vitro phenoma. The end stage mature granulocyte, the polymorphonuclear

Table 1. Production of colony stimulating factor (CSF) and prostaglandin E (PGE) by murine and human monocyte-macrophage cell lines

Cell line	CSF[a]		PGE[b]		
Mouse	Control	+ LPS	Control	+ LPS	+ CSF
WEHI-3	84 ± 4	161 ± 18	173 ± 91	1040 ± 85	863 ± 35
SK2.2	16 ± 3	91 ± 9	296 ± 13	3462 ± 149	nd
PU5.18	12 ± 2	94 ± 12	283 ± 37	2385 ± 312	nd
RAW 264	0	111 ± 1	0	305 ± 25	gceoe± 25
J774	0	128 ± 10	0	1033 ± 61	1670 ± 88
Human					
SPGcT	100 ± 3	nd	3129 ± 150	nd	nd
U937	0	0	0	0	nd

[a] Colonies/7.5×10^4 marrow cells/0.1 ml of 24 hr. conditioned medium
[b] Picograms of PGE determined by radioimmunoassay

neutrophil (PMN), has been reported to produce chalone-like activities which can suppress ^{3}HTdR uptake in proliferating myeloid cells [9] or can alter the structuredness of the cytoplasmic matrix of such cells [10]. More recently, we have shown that PMN and their products indirectly inhibit CFU-c in vitro by decreasing the production and release of CSF by monocytes and macrophages [11]. The basis of the assay for this type of inhibitory activity involves density separation of bone marrow or blood leukocytes in order to obtain populations of cells enriched for CFU-c and CSF producing cells but depleted of mature granulocytes. When cultured in agar, endogenous production of CSF results in "spontaneous" colony formation independent of an exogenous source of CSF. Addition of mature granulocytes, granulocyte extracts or granulocyte conditioned medium to such cultures reproducibly suppresses spontaneous colony formation by an inhibitory action on CSF production by monocytes and macrophages [11]. Investigations of this PMN inhibitory activity in patients with myeloid leukemia and myeloproliferative disorders have demonstrated a marked quantitative defect [12, 13]. Interestingly, this defect persisted in many patients satisfying standard criteria for complete remission and could not be correlated with chemotherapy [12]. In a study of PMN mediated inhibition of in vitro myelopoiesis in 58 patients with chronic myeloid leukemia at all stages of the disease, a double defect in negative feedback regulation was observed [13]. PMN from the patients were quantitatively deficient in inhibiting normal or leukemic colony formation but, in addition, monocytes or macrophages from leukemic patients were less sensitive than normal cells to inhibition by activity derived from normal PMN. Again this decreased responsiveness was a quantitative rather than a qualitative difference and CSF production by leukemic monocytes and macrophages of human and murine origin can be effectively suppressed by high concentrations of granulocyte inhibitor [13, 14].

The inhibitory activity in serum free human granulocyte lysates is labile and rapidly inactivated at 37°C due to protease activity [15]. Partial purification of the inhibitor was obtained by ultracentrifugation, DEAE-sephadex

chromatography, SDS polyacrylamide gel electrophoresis and isoelectric focusing. Using this purification procedure, a 1×10^6 fold purification was obtained and the inhibitor was identified as a glycoprotein with a molecular weight of 80000–100000 and isoelectric focus points of pH 6.0–6.5. This inhibitory factor was therefore similar in many respects to lactoferrin, the iron binding protein first isolated from milk and also present in epithelial secretions and mature granulocytes [16]. Purified lactoferrin from human milk was tested in the spontaneous colony assay system against normal human marrow and in the native form (8% iron saturated) was inhibitory at 10^{-15} M, indeed the fully iron saturated form inhibited at 10^{-17} M whereas the apoform (depleted of iron) was only active at concentrations $> 10^{-7}$ M [17]. Serum transferrin, a biochemically similar iron-binding protein which is antigenically distinct from lactoferrin, was only minimally inhibitory at concentrations $> 10^{-6}$ M. Separation of the granulocyte inhibitory factor and lactoferrin by isoelectric focusing confirmed that the regions of inhibitory activity corresponded in both to a pH of 6.5. In addition, the purified immunoglobulin fraction of rabbit anti-human lactoferrin antiserum, but not anti-transferrin, inactivated the capacity of both lactoferrin and granulocyte inhibitor to block CSF production [17]. It has been postulated that mature granulocytes limit CSF production by eliminating bacteria [18], however, mature granulocytes in the presence of bacterial products and in response to phagocytic challenge release large quantities of their intracellular contents of lactoferrin and suppression of CSF production under these circumstances is probably mediated via lactoferrin. The observed quantitative deficiency of lactoferrin in the mature granulocytes of patients with acute and chronic myeloid leukemia and myeloproliferative disorders [19] further substantiates our contention that this iron binding protein is involved in a primary regulatory dysfunction associated with neoplastic myelopoiesis.

III. The role of stimulatory or inhibitory activities unique to the leukemic state

The theoretical possibility exists that the growth advantage of the leukemic clone is due to selective proliferation of leukemic cells in response to a macromolecule which is not growth stimulating to normal myelopoietic cells; alternatively, leukemic cells may produce an inhibitory activity specifically suppressive to normal but not leukemic hemopoiesis. Precedents for both possibilities have been reported. Conditioned medium from human embryo cell cultures (WHE-1CM) appeared to be specific for leukemic myeloid cells since it stimulated continuous growth and differentiation in suspension culture of human acute and chronic myeloid leukemic cells but had no influence on bone marrow or blood cells from normal donors either in suspension culture or in semi-solid media [20].

Evidence for a leukemia specific inhibitory activity (LIA) has been provided by Broxmeyer et al. [21,22]. Extracts and/or conditioned medium of bone marrow or blood cells from 76 of 85 patients with acute leukemia of a variety of morphological types inhibited colony formation of normal CFU-c by 28 to 90%. The activity was not related to treatment since untreated as well as treated patients possessed cells with inhibitory activity. Extracts from cells

of 41 of 47 patients with CML and 6 of 6 patients with chronic lymphoid leukemia were also inhibitory to normal CFU-c and titration of inhibitory activity suggested that extracts obtained from patients with chronic leukemia had lower levels of inhibitor than observed in acute leukemia [21,22]. The finding that the varied leukemic states contain the same type of inhibitory activity suggests that there may be a common link between the diseases which manifest as abnormalities in different hemopoietic cell lines and which are associated with profound suppression of normal myelopoiesis. Maximum inhibitory activity was present in medium conditioned by leukemic cells after three days, exceeding the inhibitory activity obtained by direct extraction and indicating active synthesis of the inhibitor [21,22]. The specificity of the inhibitory activity resided in its capacity to inhibit normal CFU-c but not leukemic CFU-c from patients with acute and chronic myeloid leukemia. The inhibition of normal CFU-c was cell cycle specific since pulse exposure of normal marrow to high specific activity ^{3}HTdR, washing and then pulsing with leukemic cell extract resulted in no greater inhibition than after ^{3}HTdR alone.

The cell type in the bone marrow and blood of patients with leukemia which produces the inhibitory activity has been characterized as belonging to a minority population of non-adherent, non-phagocytic cells of low density (< 1.070 gm/cm^3), slowly sedimenting (2–6 mm/hr) and present in the sheep RBC rosetting population which is E^-, EAC^-, Ig^-, EA^+ and Ia^- as determined by complement cytotoxicity with rabbit anti-human Ia-like antibody [12,23]. This cell population can be distinguished from the vast majority of the leukemic CFU-c and the blast cell population of the marrow. It therefore remains unclear as to whether leukemic inhibitory activity is a direct product of a subpopulation of leukemic cells or an abnormal reactivity of non-leukemic cells imposed by the leukemic state.

3. Neoplastic Transformation of the Pluripotential Stem Cell

Short term in vitro clonal assay systems have proved invaluable in providing information on the characteristics of both normal and leukemic progenitor cells restricted to the various hemopoietic cell lineages. Such in vitro systems have the disadvantage of not supporting pluripotential stem cell maintenance and consequently can provide little information on the processes involved in the differentiation of the pluripotential stem cell into the committed compartments. Furthermore it is now very clear that in a variety of human neoplastic hemopoietic diseases such as chronic myeloid leukemia, many varieties of acute myeloid leukemia, certain preleukemic states, polycythemia vera and primary myelofibrosis, the transformed target cell is the pluripotential stem cell. Recent developments in tissue culture methodology now permit extensive proliferation of mouse pluripotential stem cells (CFU-s) for many months in a bone marrow suspension culture system [24,25]. The key to successful stem cell replication in vitro appears to be the requirement for establishing an adherent layer of bone marrow cells containing a variety of cell types representative of bone marrow stromal elements [26]. This continu-

ous bone marrow culture system provides a unique model for defining the process of leukemic transformation from the outset, particularly as it may involve phenotypic changes in pluripotential stem cells. Dexter et al. [27] have reported that infection of continuous marrow cultures with an NB-tropic pool of Friend leukemia complex virus resulted in chronic production of spleen focus forming virus (SFFV) with in vivo erythroleukemia inducing capacity. However, no evidence of in vitro erythroleukemia was obtained but sustained proliferation of CFU-s was observed in virus infected cultures and after 9–10 weeks these stem cells were atypical forming granulocytic colonies of all stages of maturation in the spleen. Using a cloned stock of NB-tropic Friend strain of helper C type virus free of SFFV (F-MuLV), we have observed that many of the phenotypic changes first reported by Dexter et al. [27] using the Friend complex were produced by the F-MuLV helper virus alone [28,29]. These changes were first evident after 10 weeks of marrow culture and were not simply attributable to virus replication since high titers of F-MuLV were produced continuously throughout the culture period. The changes involved prolonged replication of CFU-s and CFU-c with greatly increased cloning efficiency of the latter with in vitro maturation defects observed both in the colonies and cultured marrow cell population.

The evidence for transformation of pluripotential stem cells is based on the detection of replicating CFU-s in 25% of transformed agar colonies and in cell lines derived from such colonies maintained in simple suspension culture for many weeks. Unlike the observation of Dexter et al. [27], the spleen colony morphology appeared normal and lethally irradiated mice reconstituted by F-MuLV transformed bone marrow remained alive and well for many months [28,29]. Mortality from leukemia in such reconstituted mice was only evident after 5–6 months. It is highly probable that CFU-c were also target cells for F-MuLV transformation since many transformed agar colonies did not contain CFU-s but continuously replicated new CFU-c. Since CFU-c self renewal was not seen in control colonies, one phenotypic sign of CFU-c transformation may be acquisition of extensive self renewal capacity on the part of a cell population normally dependent on differentiation from a pluripotent stem cell compartment.

Phenotypic changes were also observed in cultures infected with, and replicating, SFFV in the presence of F-MuLV. These were less obvious than in the F-MuLV infected cultures suggesting the possibility that SFFV may be interfering with stem cell transformation due exclusively to the F-MuLV "helper" virus.

In contrast to the influence of F-MuLV, marrow cultures infected with pseudotypes of Kirsten sarcoma virus with Rauscher virus showed a rapid loss of stem cell and progenitor cell production associated with transformation of the macrophage component of the adherent marrow microenvironment [29]. While it is possible that a wider spectrum of potential hemopoietic target cells may exist for K-MSV and that the Rauscher helper virus may transform stem cells in a manner analogous to F-MuLV, the rapid transformation of the adherent layer precludes the persistence of stem cells and progenitor cells which might otherwise have undergone transformation.

The role of C type viruses in the development of human leukemia remains an intriguing but as yet unresolved issue. The isolation of a type-C virus (HL-23V) from human acute myelogenous leukemia cells after sustained exponential leukocyte growth in vitro [20] has further drawn attention to the need for an in vitro hemopoietic system to investigate the transformation potential of such virus isolates. We have developed two systems which may prove useful in this regard [29]. The sustained replication of human CFU-c and production of myeloid cells for some weeks in continuous human bone marrow culture strongly suggests that pluripotential stem cell replication is being supported. The system, however, remains less than optimal when compared with the duration of stem cell production in the murine system. Two obvious differences between the species have become apparent from a comparison of the cultured bone marrow. Firstly, the adherent layer established with the primary inoculum of human marrow is deficient in the foci of giant fat-containing cells so characteristic of the murine system [26]. Fat-containing cells do, however, develop within focal areas of adherent marrow cells but unlike the mouse, these latter progressively become spherical and detach [29]. A second difference is that while CSF production in the mouse marrow culture is undetectable, high levels of CSF are produced in long term human marrow culture. The accumulation of CSF may compromise the continuous replication of stem cells by favoring conversion of the culture to a predominantly macrophage morphology. Attempts to sustain human stem cell replication on murine bone marrow adherent layers proved unsuccessful presumably due to species restrictions on cell interactions and the known species specificity of various regulatory macromolecules such as CSF. As part of a phylogenetic analysis, we have been able to establish that the Tupaia, one of the most primitive living prosimians, was hematologically very similar to man but established an adherent marrow environment similar to that seen in the mouse [29]. The extensive accumulation of fat-containing foci and absence of endogenous CSF production indicated that Tupaia adherent marrow could provide a suitable microenvironment for human stem cell replication. This possibility remains to be established since all attempts to coculture human marrow on Tupaia adherent layers have been thwarted by the continuous replication at high levels of Tupaia CFU-c as confirmed by colony cytogenetic analysis. A single inoculum of Tupaia marrow appears to be sufficient to sustain continuous replication of CFU-c and differentiating granulocytic cell production far in excess of six months [29]. This production is independent of a second feeding with either fresh Tupaia or human bone marrow and indicates that this in vitro prosimian marrow culture system exceeds even that of the mouse in sustaining prolonged stem cell replication. The development of marrow culture systems in species other than the mouse should provide further insight into the control of stem cell replication and the role of these cells as potential targets for viral transformation.

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