GUEST EDITORIAL

Molecular aspects of a negative regulator of haemopoiesis

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There has been increasing interest in recent years in negative regulators of haemopoietic stem cell proliferation. Such factors, as well as being of general interest in the understanding of the regulation of haemopoiesis, may also have profound clinical implications; for example, in alleviating the neutropenia which follows destruction of the haemopoietic system by cytotoxic agents used in tumour therapy, or in the protection of stem cells in vitro during bone marrow purging prior to autologous bone marrow transplantation.

One limitation to our current knowledge of the regulators of haemopoietic stem cell proliferation is that the stem compartment is as yet ill defined and consists of a range of cell types displaying varying degrees of self renewal or differentiation potential (Schofield, 1978). The majority of ‘stem cell’ regulators have been defined on the basis of the murine Colony Forming Unit-Spleen (CFU-S) assay or equivalent in vitro assays, but it is becoming increasingly clear that these assays do not detect the most primitive haemopoietic stem cell. For efficient engraftment into irradiated recipient mice, CFU-S are required for the early transient phase and the ‘Pre-CFU-S’ compartment is required for long-term repopulation (Jones et al., 1990). Therefore, although the CFU-S (or equivalent) cell is not the most primitive in the haemopoietic system, its self-renewal capacity and multi-lineage potential serves as a valuable model system for the evaluation of stem cell regulators. It remains to be seen whether the stem cell inhibitor described below, and the other factors affecting stem cell proliferation, are also capable of altering the proliferative status of the pre-CFU-S cells.

The term ‘Stem Cell’, as used in this review, will refer to the CFU-S cell unless otherwise stated.

In the normal bone marrow, only c.10% of the haemopoietic stem cells are actively proliferating, the remaining cells being quiescent. When the murine haemopoietic system is partially ablated, for example by chemotherapeutic agents or irradiation, CFU-S cell proliferation is stimulated to regenerate the haemopoietic system and up to 60% of the pre-CFU-S cells may then be actively proliferating. Once the bone marrow is repopulated, the proliferative status of the CFU-S cells return to normal.

It has been proposed, on the basis of these and other observations, that both inhibitors and stimulators of haemopoietic stem cell proliferation must exist. Studies performed over the past 20 years have identified a number of negative regulators of haemopoietic stem cell proliferation. For the purposes of this review, and as our own studies have been dedicated to SCI/MIP-1\(\alpha\), discussion of this cytokine will dominate this review. Readers are referred to reviews by Axelrad (1990) and Graham and Pragnell (1990) for discussions on the other haemopoietic inhibitors.

SCI/MIP-1\(\alpha\) and the inhibition of stem cell proliferation

Studies by Lord, Wright and colleagues in the mid seventies identified an activity in normal bone marrow extracts which inhibited murine haemopoietic CFU-S cell proliferation apparently by holding stem cells close to the G_1/S phase transition point (Lord et al., 1979; Wright et al., 1980, Lord & Wright 1980). This stem cell inhibitor (SCI) was produced by bone marrow derived macrophages, and was specific for the stem cell compartment, having no inhibitory effects on more mature progenitors.

We have recently developed an in vitro assay for a cell with properties in common with the day 12 CFU-S cell (Pragnell et al., 1988; Lorimore et al., 1990). This assay has been used to characterise and purify an inhibitor of CFU-S proliferation (Graham et al., 1990) which we have called Stem Cell Inhibitor (SCI).

Primary sequence analysis revealed that murine SCI is identical to a previously described cytokine, Macrophage Inflammatory Protein 1\(\alpha\) (Davatelas et al., 1988), a member of a large family of related cytokines. This family is defined on the basis of sequence homology and on the presence of four cysteines which have been positionally conserved (for a review see Wolpe & Cerami, 1989). A subset within this family includes the basic cytokine MIP-2, Platelet Factor-4 (PF-4), \(\beta\)-thromboglobulin, Interleukin-8 and melanoma growth stimulatory activity (MGSA) proteins. MIP-2 and PF4 are chemotactic for polymorphonuclear cells (see Wolpe & Cerami, 1989).

SCI/MIP-1\(\alpha\) is a small heparin binding peptide with a molecular weight of 8 kD although the molecule readily forms large non covalent aggregates with molecular weights in excess of one million daltons. Intriguingly, a related peptide (70% homologous at the amino acid level), MIP-1\(\beta\) (Wolpe & Cerami, 1989), which copurified with MIP-1\(\alpha\) displays no inhibitory activity at the concentrations tested (Graham et al., 1990). More recent reports suggest that SCI/MIP-1\(\alpha\), MIP-1\(\beta\) and MIP-2 can stimulate progenitor cell proliferation, but only in conditions where growth factor concentrations are limiting in in vitro progenitor assays (Broxmeyer et al., 1990).

The human SCI/MIP-1\(\alpha\) homologue is also a peptide with a molecular weight of approximately 8 kD and forms large self aggregates. It is equally effective as a CFU-S proliferation inhibitor and we are currently using the human SCI/MIP-1\(\alpha\) in preclinical trials designed to test its efficacy in reducing myelotoxicity during drug treatment of mice.

The inhibitory activities of SCI/MIP-1\(\alpha\) are not confined to the haemopoietic system. We have shown both human and murine SCI/MIP-1\(\alpha\) to be active in inhibiting clonogenic epidermal cells (Parkinson & Graham, unpublished results), although it is not yet clear whether this is a direct or indirect effect on the primary epidermal cells. The source of SCI/MIP-1\(\alpha\) in vivo in the skin remains to be determined, however, two possibilities are being considered. In the normal mouse skin epidermal proliferation unit (EPU), a Langerhans cell, which is of monocytic/lymphoid origin and expresses SCI/MIP-1\(\alpha\), is in close proximity to the slow cycling epidermal keratinocyte and may be involved in establishing the proliferative hierarchy observed. Alternatively, during skin wounding and/or inflammation, local infiltration of macrophages and T-cells may produce SCI/MIP-1\(\alpha\) although the functional implications of such production would have to be investigated. The availability of probes allowing in situ or immunocytochemical analysis should allow us to investigate these possibilities further.

SCI/MIP-1\(\alpha\) gene structure

The mouse MIP-1\(\alpha\) protein was originally purified from endotoxin stimulated macrophages and sequenced. This led
to the isolation of a cDNA alone (Davatelis et al., 1988) and subsequent isolation and sequence of a single encoding gene (Grove et al., 1990). At least three independent groups have cloned human cDNA's which have turned out to be homologous to human SCI/MIP-1x: Obaru et al. (1986) cloned the LD78 cDNA using differential hybridisation of tumour promoter stimulated human tonsillar lymphocytes; Zipfel et al. (1989) cloned the cDNA (pAT 464) from mitogen stimulated peripheral blood T cells; and Forsdyke (1985) cloned the cDNA (GOS19-1) from leucin stimulated cultured blood mononuclear cells.

Southern blot analyses of EcoR1 digested human DNAs probed with LD78, or pAT 464 cDNA sequences revealed 1 – 3 bands depending on the DNA source (Irvine et al., 1990, Nakao et al., 1990). Three groups have independently cloned at least three human SCI/MIP-1x-related genes, at least two of which are linked: LD78x/pAT 464.1/GOS19-1 and LD78x/pAT 744.1/GOS19-2 (Nakao et al., 1990; Irving et al., 1990; Blum et al., 1990). The pAT 464.1 and pAT 744.1 genes are separated by 14 kb and are transcribed from opposite strands of DNA. This suggests that the two genes recently arose by gene duplication and divergence which included the insertion of an Alu repetitive sequence into the 5' flanking sequence of the GOS19-2/LD78x gene (Nakao et al., 1990; Blum et al., 1990). A third member of the SCI/MIP-1x family, most closely related to LD78, LD78x and GOS19-3 (Nakao et al., 1990; Blum et al., 1990; Irving et al., 1990), has been isolated as a putative human MIP-1B cDNA homologue (Act-2, Lipes et al., 1988) but these have yet to be fully characterised.

The mouse SCI/MIP-1x and the related TCA-3 and MIP-1B genes are clustered on chromosome 11 within 5 cM of the p53 (proximal) and Hox-2 (distal) loci. In humans, SCI/MIP-1x (LD78) and the related TCA-3 and MIP-1B genes are clustered on chromosome 17 (q11-121) (Irvine et al., 1990). However, although the murine MIP-2 gene also maps to chromosome 11, members of the human MIP-2 gene family (PF4 and MGSA), map to chromosome 4q13-22 (Wolpe & Cerami, 1989).

The human SCI/MIP-1x gene maps near to sites of genetic lesions (17q11-q21) associated with a number of disorders. For example, (a) the acute promyelocytic leukaemia (APL) t(15;17) (q22,q12-2) translocation, involves a specific breakpoint (17q21.1) at the retinoic acid receptor-a gene (de The et al., 1990); (b) von Recklinghausen neurofibromatosis (NF1), an autosomal dominant disease, where the NF1 gene encodes a protein containing ras GTPase activity (Xu et al., 1990); and (c) as a result of heterozygosity (LOH) in breast cancer (Cropp et al., 1990). Although it is unlikely that the SCI/MIP-1x gene is itself directly involved in the cellular transformation process, nearby genetic lesions which activate proto-oncogenes or inactive suppressor genes may also coincidentally lead to aberrant SCI/MIP-1x gene expression. The up-regulation of SCI/MIP-1x gene expression has been detected in the peripheral blood of a number of ANLL and ALL patients (Yamamura et al., 1989) and in haemopoietic cells derived from patients with aplastic anaemia and myelodysplastic syndrome (N.S. Young, personal communication). It would be interesting to determine whether aberrant SCI/MIP-1x protein expression contributes to the suppression of normal haemopoiesis in these patients, and whether the SCI/MIP-1x gene locus on chromosome 17 has been disturbed in these neoplasia.

**SCI/MIP-1x gene expression**

SCI/MIP-1x protein was first described as a haemopoietic stem cell inhibitor activity in bone marrow extracts (Lord & Wright, 1980) and monoclonal antibodies to human SCI/MIP-1x mRNA is barely detectable in normal bone marrow, but is readily detectable in cultured bone marrow macrophages (A. Reid unpublished results). More recent studies indicate that SCI/MIP-1x gene expression is only detectable in a limited number of haemopoietic cell lineages – macrophages (Davatelis et al., 1988; Wolpe & Cerami 1989; Obaru et al., 1986, Yamamura et al., 1989), epidermal Langerhans cells (K. Parkinson unpublished results), activated T-cells (Yamamura et al., 1989) and mast cells (Gordon et al., 1990). One report describes the detection of SCI/MIP-1x gene transcripts in phorbol ester treated primary cultured human fibroblasts and a human glioma cell line (U105MG) (Nakao et al., 1990), but it is unclear whether this represents cross-hybridisation of the human LD78 probe to transcripts from a related member of the MIP multigene family. Structural analysis of the nuclear murine SCI/MIP-1x gene in fibroblast and epithelial cell lines suggest the gene is in an inactive conformation, consistent with the inability to detect SCI/MIP-1x mRNA by Northern blot or Polymerase Chain Reaction (PCR) analyses (M.P. unpublished results).

In addition to the apparent tissue-specificity of SCI/MIP-1x gene expression, the molecular mechanism(s) regulating its expression are beginning to be elucidated. Published and unpublished data from this and other laboratories, and a comparison with analyses of other cytokines involved in haemopoiesis and the immune response, indicate that SCI/MIP-1x gene expression is regulated at the levels of transcription, mRNA stability, mRNA processing and translation. For example, sequence analysis of the murine (Davatelis et al., 1988) and human (Forsdyke, 1985; Obaru et al., 1986; Zipfel et al., 1989) SCI/MIP-1x cDNAs revealed a number of conserved (TATTGT) motifs in the 3' untranslated region of the mRNA which have been implicated in the modulation of mRNA stability of a number of other cytokine mRNAs (Caput et al., 1986; Shaw & Kamen, 1986). Similarly, as mentioned above, SCI/MIP-1x mRNA is super-induced during endotoxigenic (lipopolysaccharide, LPS) stimulation of murine macrophages (Davatelis et al., 1988), and is super-induced in human T-cell lines by phorbol esters (PMA) and/or PHA and cyclohexamide (Obaru et al., 1986; Yamamura et al., 1989; Blum et al., 1990). Interestingly, a basal level of SCI/MIP-1x mRNA is detected in unstimulated cultured murine macrophage (Graham et al., 1990; Davatelis et al., 1988) and mast cell lines (Gordon et al., 1990, and M.P. unpublished results), whereas it is undetectable in unstimulated human monocyte (U937) and T-cell (Jurkat) lines although it can be induced by PHA and/or PMA (Obaru et al., 1986; Yamamura et al., 1989; Blum et al., 1990).

Sequence comparison of the proximal promoters of the human and mouse SCI/MIP-1x genes with those of other cytokine genes such as GM-CSF, have revealed a number of conserved potential transcription factor binding sites. These include potential NF-kB (related to the c-Rel proto-oncogene (Bordier et al., 1990), and NF-κB-like binding sites, which may be shared with the NF-kB family of proteins, (Shannon et al., 1990)), API (encoded by the c-fos and c-jun proto-oncogenes, for review see Abate & Curran, 1990) and PU1 (a member of the c-ets family of proto-oncogenes (Klemsz et al., 1990) binding sites. These transcription factors are all nuclear proto-oncogenes which have been implicated in the early response to mitogenic stimuli (e.g. phorbol esters and API1 in cell proliferation. This implies that SCI/MIP-1x gene transcription is regulated as a function of cell proliferation/activation, and as it is a negative regulator of stem cell proliferation it is tempting to speculate that one role of SCI/MIP-1x, particularly that produced by macrophages in the bone marrow, is as a classical negative feedback factor. Furthermore, it raises the possibility that the activation of the nuclear proto-oncogene(s) in certain neoplasias may lead to aberrant SCI/MIP-1x gene expression.

**Concluding remarks**

It is clear that SCI/MIP-1x gene expression is specific to a limited number of haemopoietic cells, is regulated at both transcriptional and post-transcriptional levels, and may also be regulated at the translational and post-translational levels.

There is ample evidence to suggest that the control of haemopoietic stem cell proliferation occurs at the level of the
stromal micro-environment. It is therefore necessary to examine the localisation of SCI/MIP-1α protein in the bone marrow extra-cellular matrix as it is clearly synthesised and released by bone marrow cells, including macrophages. One obvious approach is to utilise in situ hybridisation and immunocytochemical techniques, although to date it has proven very difficult to demonstrate the presence of either peptide or mRNA transcripts of other known cytokines in normal bone marrow or longer-term marrow cultures. If SCI/MIP-1α is also expressed during embryogenesis it is yet undetectable by in situ hybridisation analysis (Hastie, personal communication), in contrast to TGF-β which is readily detectable in embryonic tissue.

Another approach to elucidate the physiological relevance of a cytokine is by gene inactivation using homologous recombination. However, as SCI/MIP-1α is part of a multi-gene family and there appears to be a redundancy of function between various haemopoietic growth factors, single gene inactivation may not be particularly informative, and cross-breeding of mice with a range of inactivated genes may well provide an exciting approach to the problem. Alternatively, studies on the tissue distribution of the SCI/MIP-1α receptor would provide invaluable information on the physiological role of the cytokine, but the biochemical properties of the purified peptide is currently hampering progress in binding studies.

The clinical potential of SCI/MIP-1α is obvious. We now have evidence that inoculation of mice with SCI/MIP-1α in vivo leads to a dose dependent reduction in the cycling status of CFU-S/CFU-A cells (D. Dunlop, personal communication). Experiments are now underway in a number of laboratories to evaluate various therapeutic drug protocols.

The possibility that SCI/MIP-1α contributes to the suppression of stem cell proliferation in certain neoplasias remains to be explored. In parallel, it would be of significant interest if aberrant SCI/MIP-1α gene expression could be linked to specific chromosomal translocations or the activation of certain nuclear proto-oncogene(s). Thus, whilst there are a number of intriguing observations concerning SCI/MIP-1α, a number of detailed studies are required before a clear picture of its physiological role will emerge.

The authors work was supported by a grant to the Beatson Institute from the Cancer Research Campaign.

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