A Comparative Study of Growth Stimulating and Inhibiting Activities Secreted by a Series of Stromal Cell Lines

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A Comparative Study of Growth Stimulating and Inhibiting Activities Secreted by a Series of Stromal Cell Lines

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Abstract

A series of stromal cell lines were intercompared for their ability to secrete into the growth medium under serum-free conditions factors that stimulate/inhibit haemopoiesis. Although all of the cell clones produced colony stimulating activities for bone marrow cells, we observed an inverse relationship between the ability of the stromal cells to support haemopoieisis and the titer of the stimulating factors it produced. The secretion of inhibitory activities also varied among the members of the stromal cell series. The stromal clone that supports haemopoiesis was a poor producer of secreted inhibitory activities. The latter are transforming growth factor-β (TGFβ) as well as additional undefined factors. It is suggested that the combination of low expression of differentiation inducers and secreted inhibitors contribute to the stromal cell ability to support long-term haemopoiesis.

Results and Discussion

In the past, we have proposed that stromal cells provide two essential components of the regulatory system governing haemopoiesis (1-3). One is

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a "differentiation restraining activity" that is closely associated with the cell surface. This activity blocks the option of differentiation and leads the stem cell to divide and renew itself. The second stromal activity is a "bone marrow resident colony stimulating factor". This stromal factor affects early stem cells. By itself it is a rather poor stimulator of colony formation but it drives stem cell differentiation and formation of giant colonies by synergizing with other stimulating factors. In contrast to the differentiation restraining activity, the resident factor was shown to be released from the cells in greater titer and could travel through relatively thicker agar barriers to affect target haemopoietic stem cells. Nonetheless, neither stromal activities could be found in significant titers in conditioned media and were therefore studied using co-culture systems (1-3). The differentiation restraining factor that should account for stem cell renewal has not been identified to date. On the other hand, c-kit ligand (stem cell factor) (4) conforms to the description of what we have called "bone marrow resident factor". Indeed it affects early stem cells, it is a product of stromal cells and is a potent synergistic factor to a variety of colony stimulating factors (CSFs) and interleukins.

The question raised is whether the molecules discussed above are sufficient to explain all processes occurring in the haemopoietic system. We have formulated a revised model of haemopoiesis, the "theory of restrictins", that predicts the existence of additional molecules participating in the regulation process. These are mainly lineage specific inhibitors that are expected to complement the poor lineage specificity of growth and differentiation factors. Thus, GM-CSF, for example, induces the differentiation of granulocytes/macrophages as well as other members of the haemopoietic system. The uniform micro-domains within the micro-environment of the bone marrow where cells of a single phenotype reside, such as erythroid islands or granulocyte nests, may be maintained by restrictins that block the accumulation of cells other than the required ones. Among these putative molecules one factor, that specifically kills plasma-cytoma cells (restrictin-P), was studied in greater detail (5, 6). The above-mentioned differentiation restraining factor may be such a restrictive factor that blocks all options of differentiation and leaves the stem cell with the sole option of self renewal. It is equally possible, however, that stem cell renewal is mediated by a novel inducer cytokine in the absence of CSFs. Stromal cells would then be expected to secrete this cytokine, rather than the well-known lymphokines and monokines.

Stromal cell lines from the bone marrow have been subject to many investigations aimed at understanding the regulation of haematopoiesis (7). Many of the clonal populations of stromal cells share properties. However, a series of cell lines derived in our laboratory provide a unique experimental tool since they markedly differ in their morphology, extracellular matrix composition, cytoplasmic enzymes and, most importantly, in their biological effects on haemopoietic target cells (7-9). Thus, the 14F1.1 stromal cell clone of endothelial-adipocytes supports long-term

haematopoiesis (10), whereas other stromal clones do not. Figure 1 demonstrates the formation of cobblestone areas containing cells of early haemopoietic phenotype on 14F1.1 monolayers following seeding of bone marrow cells from 5-fluorouracil treated mice. This phenomenon does not occur when similar bone marrow cells are cultured with the other cell lines. The question raised is whether the lack of ability to induce haemopoiesis by some of the stromal cell clones is due to inability to secrete an essential cytokine or to excessive production of inhibitory substances. It was, therefore, of interest to intercompare the various cell clones for their ability to elaborate factors that either stimulate or inhibit haemopoeisis.

Figure 1 Photomicrograph showing a confluent layer of 14F1.1 cloned stromal cell line seeded with 2 x 10^5 bone marrow cells from day 2 post 5-fluorouracil treatment. Cobblestone area of young myeloid cells is observed.

To minimize participation of serum components in the induction of biological activities in stromal cells, we used a serum replacing medium (DCCM-1) to prepare conditioned media from confluent layers of stromal cells. Figure 2 shows the results of experiments in which colony stimulating activity (CSA) was measured in these conditioned media using a standard bone marrow assay. Among the various cell lines tested, the 14F1.1 cell clone exhibited the lowest titer of CSA while the MBA-1.1.1 clone that does not support haemopoiesis had the highest titer. In addition, this clone and MBA-2.1 cells elaborated inhibitory activities that yielded a bell-shaped dose response curve rather than a plateau plot obtained with the other conditioned media studied. One possible reason for the inhibition

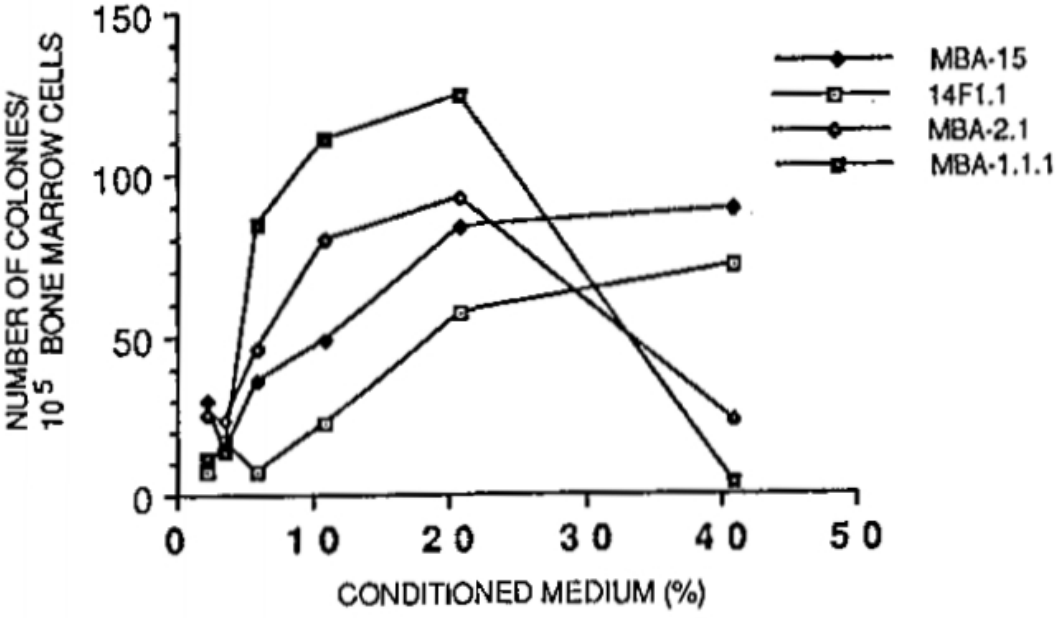

Figure 2 Colony stimulating activity in serum-free conditioned media from various stromal cell lines: bone marrow cells at 10^5/plate were seeded in methylcellulose semisolid medium with the indicated concentrations of conditioned media. The stromal cell line designations are indicated in the figure. Myeloid colonies were counted at day 8.

seen at relatively high concentration of conditioned medium could be the presence of inhibitory cytokines. A common candidate is TGFβ that has been reported to affect haemopoietic cell growth (11, 12). The inhibition could not, however, be a result of secretion by the stromal cells of TGFβ only, since the latter was shown to effectively inhibit the formation of colonies by very early myeloid progenitors while GM-CFC are virtually refractory (11, 12). The stimulating activity of the various stromal cell lines was mainly due to production of colony stimulating factor-1 (CSF-1) as demonstrated by use of CSF-1 dependent cell line 14M1.4[8] (fig. 3). Other cytokines such as interleukin-1 (IL-1) (fig. 4), IL-2 (fig. 5), IL-3 (fig. 6) and IL-4 (fig. 7) were not produced by 14F1.1 cells. Also, G-CSF and GM-CSF do not appear to contribute to the colony stimulating activity of 14F1.1 cells since the messenger RNA of these cytokines was not detected in these cells and factor dependent cell lines addicted to the corresponding cytokines were not induced by conditioned media form 14F1.1 (10). These stromal cells may express c-kit ligand which, if produced, is secreted under these conditions at a very low titer. However, this stem cell factor was shown so far to synergize with colony stimulating factors in differentiation processes and to potentiate early stem cells in a manner similar to the effects of IL-6 or IL-11. Both these functions do not conform with a putative role of this molecule as a stem cell renewal factor.

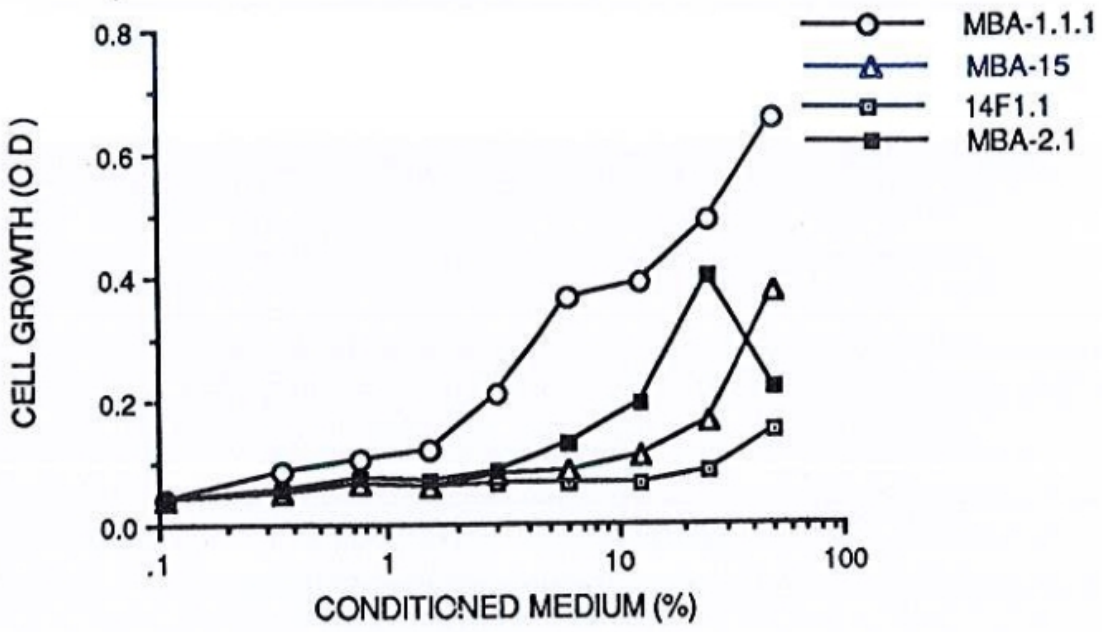

Figure 3 CSF-1 in media conditioned by various stromal cell lines. 14M1 cells, that require CSF-1 for growth, were seeded at 2×10^4/ml in microtiter plates (100ml/well) with the indicated concentrations of conditioned media. Cell growth was determined at day 4 of culture, using the MTT colorimetric assay.

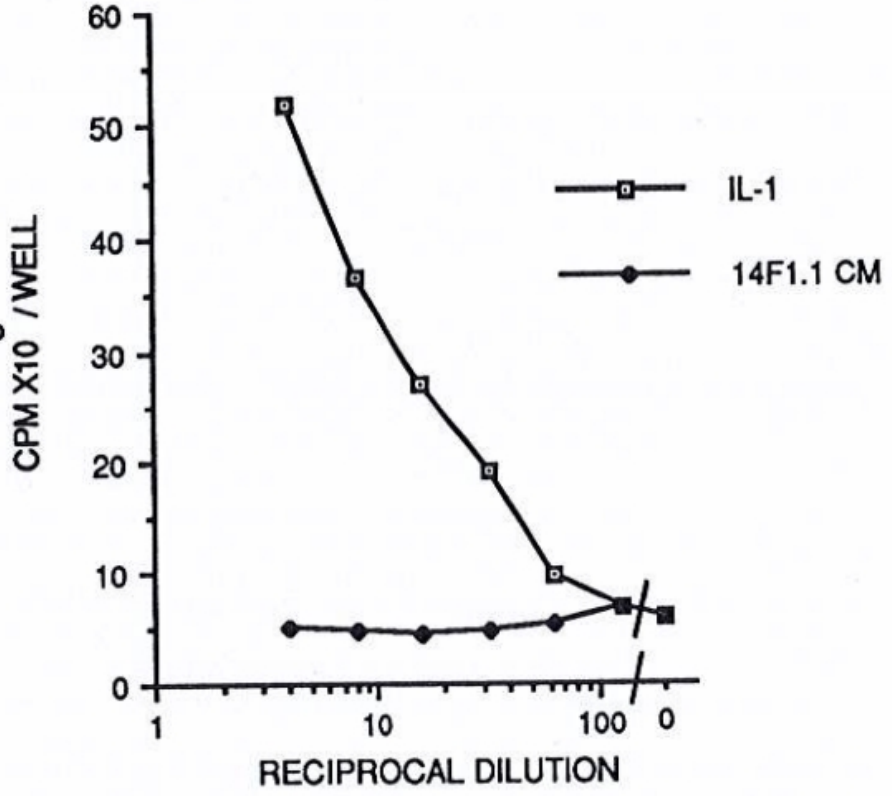

Figure 4 Examination of IL-1 activity in serum-free conditioned medium from 14F1.1 cells tested in LBRM 33-1A5/CTLL combined assay. IL-1 used as control is from thioglycollate induced peritoneal macrophages.

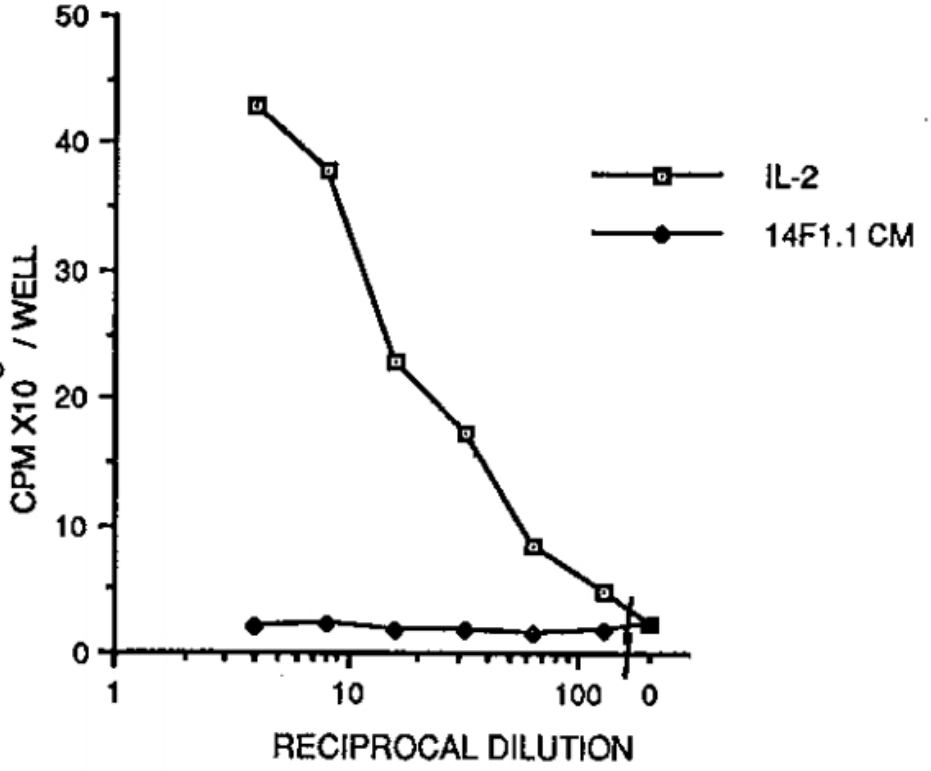

Figure 5 Examination of IL-2 activity in serum-free conditioned media from 14F1.1 cells tested by IL-2 dependent T-cells (CTLL). Concanavalin A stimulated spleen cell conditioned medium served as a source of IL-2 in the positive control group.

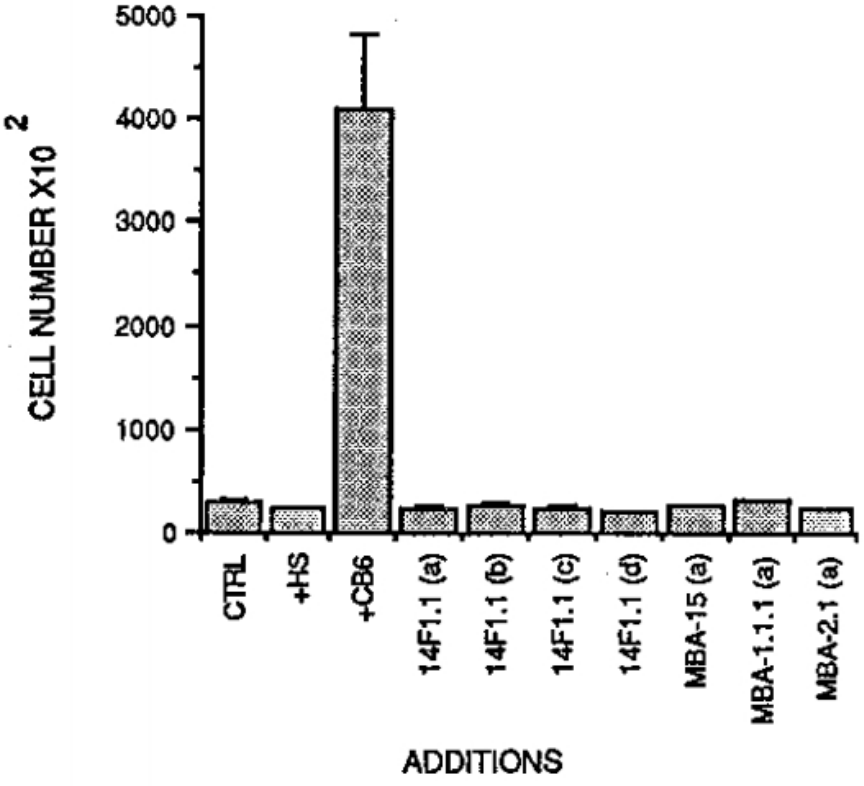

Figure 6: Caption opposite

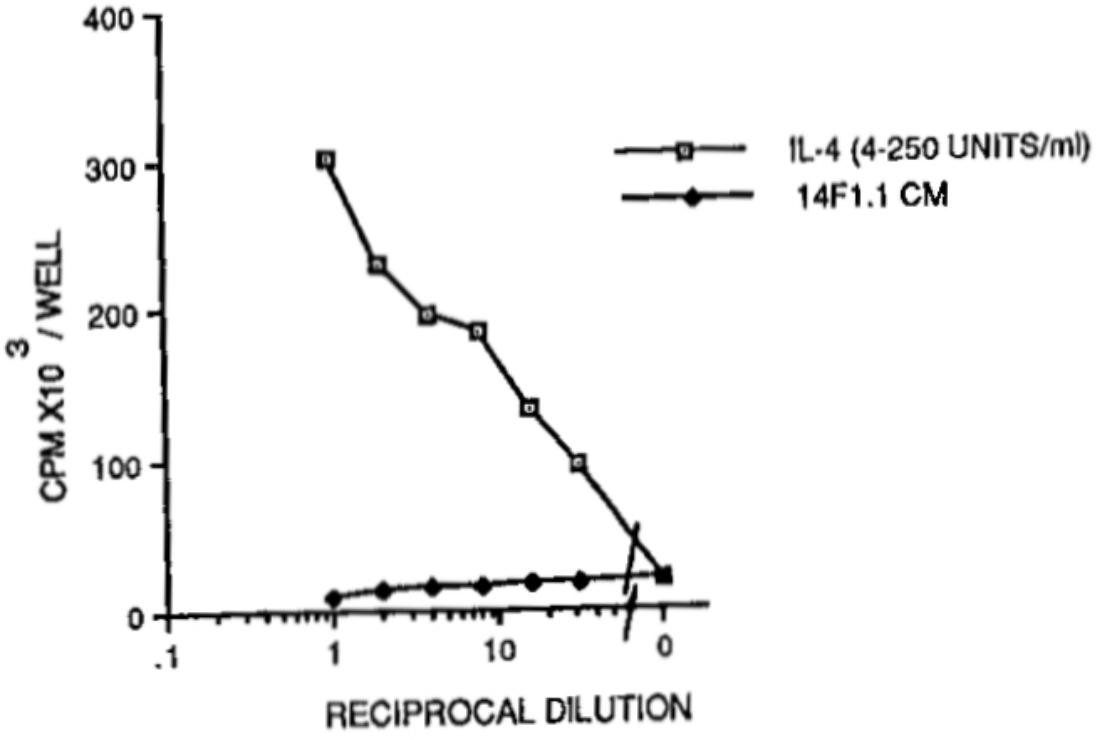

Figure 7 Examination of IL-4 activity in serum-free conditioned medium from 14F1.1 cells tested by CT-4S, IL-4 dependent T-cell line. Recombinant IL-4 was used as positive control.

One difficulty in the monitoring of cytokine gene expression, using factor dependent cell lines to follow the secretion of biologically active protein, is that the producer cell may also make molecules that inhibit the growth of the detector cell, thus yielding a false negative result. To ensure that this did not occur in the above experiments, we tested the effect of conditioned media from the different cell clones on the factor dependent cell line MC/9. These cells were grown with a plateau concentration of IL-3 that efficiently and specifically promotes MC/9 cell growth. As seen in Fig. 8, conditioned media from 14F1.1 and MBA-15 cells did not interfere with the growth of MC/9 cells but conditioned media from the other cell lines, MBA-2.1 and MBA-1.1.1, clearly did. The same cell lines also inhibited the growth of M1 myeloid cells (fig. 9). Since the latter is well known to be sensitive to TGFβ, we used neutralizing antibodies to this cytokine and found that TGFβ accounts for at least a part of the growth inhibiting activities observed (results not shown).

← **Opposite: Figure 6** Examination of IL-3 activity in serum-free conditioned media from various stromal cell lines was tested by MC/9 IL-3 dependent cell line. MC/9 cells were seeded at 10^3/ml in serum-free medium (CTRL) supplemented with 10% HS (+HS) and various additions indicated. Addition of IL-3 (+CB6 conditioned medium) allows extensive cell proliferation. Conditioned media were added at the following concentrations: (a) 100%, (b) 50%, (c) 25%, (d) 10%.

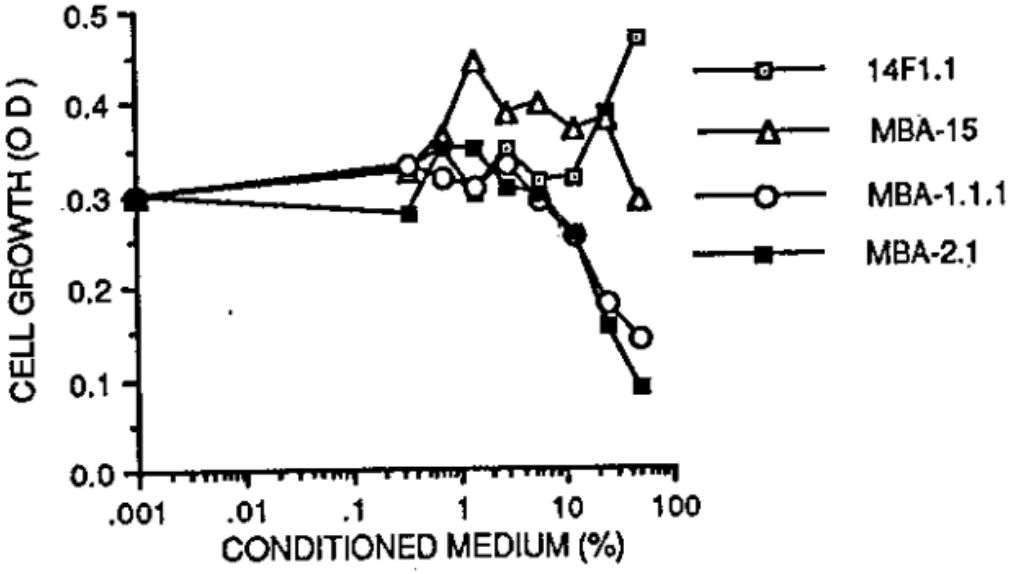

Figure 8 Inhibitory activities for MC/9 cells in media conditioned by various stromal cells. MC/9 cells (10^3/well) were seeded into microtiter plates with the indicated concentrations of conditioned media, supplemented with 10% HS and 20% IL-3 containing conditioned medium. Cell growth was determined at day 4 of culture, using the MTT colorimetric viability assay.

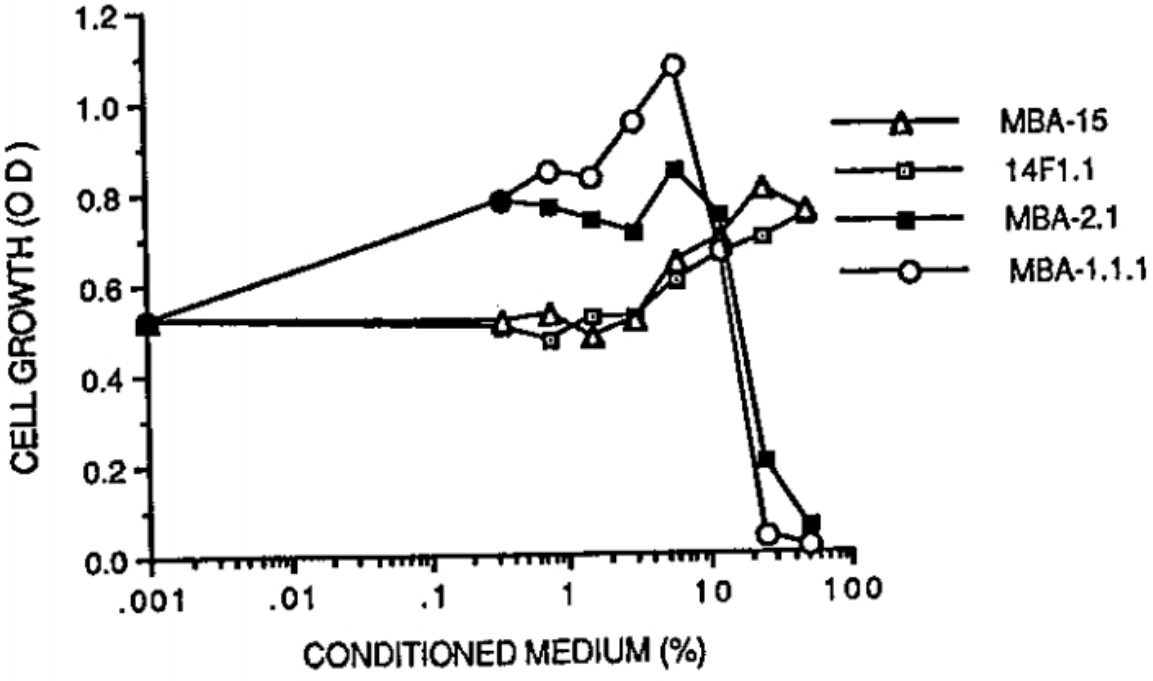

Figure 9 Inhibitory activity for M1 cells in media conditioned by various stromal cells. M1 cells were seeded at 2 x 10^4/ml in microtiter plates (100ml/well) with the indicated concentrations of conditioned media supplemented with 10% FCS. Growth was determined using the MTT colorimetric assay.

Table 1 summarizes the results presented above in a schematic form. It is shown that the ability to support long-term haemopoiesis was confined to one clone only, whereas the ability to secrete CSF was common to all cell lines. The titer of stimulating activity detected, varied considerably and was the highest in the MBA-1.1.1 fibroblast cell line (relative activities of the various cell line were: MBA-1.1.1 < MBA-2.1 < MBA-15 < 14F1.1). Also, the production of inhibitory activities varied considerably such that 14F1.1 and MBA-15 had no growth inhibiting effects, whereas the other cell lines were very potent in producing inhibitors for both M-1 and MC/9 cells, as well as to normal myeloid progenitors. It is significant that the cell that supports haemopoeisis (14F1.1) is the poorest producer of stimulatory activities and also a poor producer of inhibitors. Is this the feature that makes it a suitable substratum for the proliferation and renewal of stem cells? Conversely, are there specific molecules that are elaborated by the 14F1.1 clone and not by the other cell lines that account for the ability to support haemopoiesis? The answer for these questions should await further investigations.

Table 1 Stimulatory and inhibitory activities in conditioned media from a series of stromal cell lines

Cell lines		Renewal Activity	Differentiation activity*	Inhibitory activity
Designation	Type			
14F1.1	Endothelial-adipocyte	+	+	–
MBA-15	Osteoblastic	–	+	–
MBA-2.1	Endothelial-like	–	+++	+
MBA-1.1.1	Fibroblastoid		++++	+

+ = Active; – = not active * The titer of differentiation activity is presented in arbitrary manner, and relates to data in Figures 2 and 3.

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