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Regulation of the proliferative potential of cord blood long-term culture-initiating cells (LTC-IC) by different stromal cell lines: implications for LTC-IC measurement

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Abstract

Background and Objective. **Long-term culture-initiating cells (LTC-IC) are the best available approximation to an *in vitro* assay of stem cells in humans although they still represent a heterogeneous population in terms of proliferative capacity and sensitivity to different growth factors. Human umbilical cord blood (CB) is rich in hemopoietic progenitor cells, as measured by clonogenic assays and contains stem cells capable of reconstituting the marrow after ablation in clinical transplantation. We evaluated the influence of culture conditions on the *in vitro* behavior of LTC-IC from CB.**

Design and Methods. **LTC-IC were evaluated in long-term cultures, comparing two types of murine stromal cell lines: M2-10B4 and M2-10B4 transfected with cDNAs for human G-CSF and IL-3.**

Results. **Two and five fold higher numbers of terminally differentiated cells were produced during nine weeks of culture of CB mononuclear or $CD34^+$ cells respectively, in cultures containing a M2-10B4 IL-3 G-CSF cell line compared to cultures containing the parental cell line. Likewise, a higher number of colony-forming cells (CFC) were detected in the supernatant of cultures with the transfected cell line. In contrast, the number of CFC generated within the stromal layer, after 5 or 9 weeks of culture, was significantly higher in cultures on M2-10B4 cells than those on M2-10B4 IL-3 G-CSF.**

Interpretation and Conclusions. **Our results show that the proliferative capacity of CB LTC-IC can be strongly influenced by culture conditions and that the frequency of LTC-IC estimated using these cell lines as stromal support is not identical.**

Key words: cord blood, LTC-IC, proliferative potential

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Much of the current knowledge concerning the regulation of normal hemopoiesis and its alteration in disease has resulted from advances in techniques for culturing human hemopoietic cells *in vitro*. Long-term bone marrow cultures (LTC) *in vitro*, by allowing the formation of adherent layers that are structurally and functionally similar to the *in vivo* hemopoietic microenvironment, are able to support the growth and differentiation of hemopoietic cells for several months. This is, therefore, a system in which early stages of cell development and hemopoietic and stromal cell interactions are accessible for analysis.

LTC detects a population of cells, long-term culture-initiating cells (LTC-IC), which in mice has been demonstrated to contain the putative stem cells and in humans was originally defined as cells with the capacity to generate colony-forming cells (CFC) for more than five weeks on a supportive stroma.

Several studies have demonstrated that stromal cell lines of human or murine origin can also support LTC-IC and allow the generation of CFC for many weeks.[1-5] It has been reported that the murine fibroblast M2-10B4 cell line can support the maintenance of LTC-IC from human bone marrow as effectively as standard human marrow adherent layers.[1] The same cell line, engineered by transfection with cDNAs for human granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), was shown to increase the production of nonadherent cells (granulocytes and macrophages) and CFC when used as feeders in LTC, and to enhance the maintenance of LTC-IC without a decline in their proliferative potential.[1,6]

The incidence of LTC-IC may be estimated using a limiting dilution assay,[7,8] This is a technically demanding and time consuming assay. However an estimation of the LTC-IC content may be obtained by using standard LTC and measuring the CFC output at certain endpoints, usually 5 or 8 weeks.[9,10] We have recently demonstrated that scoring LTC-IC from apheresis samples, in limiting dilution using the M2-

10B4 cell line or normal human bone marrow stroma, can lead to equivalent results when CFC generation after 5 weeks of culture is the endpoint. However, when scoring the cobblestone area forming cells only, a higher frequency of LTC-IC was estimated in cultures utilizing the M2-10B4 cell line.[11]

Here we have compared the LTC-IC content in mononuclear cells (MNC) and in the CD34$^+$ enriched cell population from CB in parallel long term cultures using either the parental M2-10B4 or the M2-10B4 IL-3 G-CSF transfected cell lines as the supportive stroma.

Materials and Methods

Collection and preparation of cells

Umbilical cord blood samples were collected after informed consent from full-term normal deliveries. Samples were collected in sterile tubes containing 4-6% CPD (citrate, phosphate, dextrose), transported at room temperature and processed within 24 hours after collection. Before separation the samples were diluted 1/1 with phosphate buffered saline (PBS), and the mononuclear cells (MNCs) isolated by density gradient centrifugation (Lymphoprep, 1.077 g/mL, Nycomed; Oslo, Norway) at 400 g for 25 minutes. The MNCs at the interface were collected and washed in PBS containing 0.5% bovine serum albumin (BSA).

Isolation of CD34$^+$ cells

After the MNCs had been washed and counted, the CD34$^+$ fraction was isolated using MiniMACS columns and a CD34 Isolation Kit, as described elsewhere (Miltenyi Biotec; Bergisch Gladbach, Germany).[12] Briefly, MNCs were incubated with a blocking agent and a CD34 antibody (QBEND/10) from the Isolation Kit for 15 minutes at 4° to 8°C. Cells were then washed once in a cold solution of PBE (PBS, 0.5% BSA and 5 mM EDTA). The cell pellet was then resuspended in a small volume of PBE and incubated with microbeads conjugated to an anti-mouse antibody. Target cells were isolated by passing the entire cell suspension through a MiniMACS column in a magnetic field in which the CD34 microbead-labeled cells were retained on the column. The CD34$^+$ fraction was recovered by releasing the magnetic field and flushing the target cells from the column. The purity of cells isolated was between 60% to 90% as determined by flow cytometric analysis.

Flow cytometric analysis

One-two $\times 10^5$ MNCs and enriched CD34+ cells were labeled with a CD34 antibody (HPCA-2; Becton Dickinson, San José, CA, USA) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Appropriate isotype matched controls conjugated with the same fluorochrome were used. Cells were incubated with antibody for 20 minutes and then washed twice with PBS/1% BSA. Labeled cells were analyzed on a FAC Scan™ (Becton Dickinson, San Jose, CA, USA) flow cytometer equipped with an argon-ion laser tuned at 488 nm. At least 20,000 events were acquired for both control and positive samples. Data files were analyzed with LYSYS II software (BDIS).

CFC-MIX assays

CFC-MIX assays were performed as previously described.[13,14] Briefly, cells were added to a 1 mL mixture containing 30% fetal calf serum (FCS), 10% deionized BSA, 10% 5637-conditioned medium (from the 5637 EJ bladder carcinoma cell line), 2 units of erythropoietin (Boehringer, Mannheim, Germany) and 1.35% methylcellulose. Cells were plated in triplicate and incubated for 14 days at 37°C in an incubator with 5% CO_2 in air. Colonies of granulocyte-macrophage colony-forming cells (GM-CFCs) were assessed according to established criteria after 14 days of incubation.[15]

Stromal cell lines

Cell lines were kindly provided by Dr. C.J. Eaves, Terry Fox Laboratory, Vancouver, Canada. M2-10B4 and M2-10B4 IL-3 G-CSF G418^r stromal cells were maintained in RPMI-1640 medium (GIBCO, Grand Island, NY) and 10% FCS and subsequently passaged as continuous cell lines. Transfected cells were selected by adding 0.4 mg/mL G418 to the culture medium. Feeders were prepared by seeding 1-5$\times 10^5$ cells in 25 cm^2 tissue culture flasks and maintained at 37° C and 5% CO_2 in air. At confluence, all feeders were irradiated (60 Gy delivered from a ^{137}Cs γ-rays source) to halt further proliferation.

Long-term cultures (LTCs)

The test inocula were resuspended in LTC medium containing Iscove's modified Dulbecco's medium (IMDM) at 350 mOsM/kg, 10% horse serum, 10% FCS and 5×10^{-7} M hydrocortisone (Sigma). The feeder layers were then loaded with 10 mL of LTC medium containing either 10^6 MNCs or 5×10^4 enriched CD34$^+$ cells per 25 cm^2 flask. The cultures were maintained at 33°C with weekly replacement of half of the medium. All the experiments were performed in parallel, inoculating aliquots of the same cell population in flasks containing either the M2-10B4 or the transfected cell line. Aliquots of non-adherent cells were assessed weekly over a period of 9 weeks for content of clonogenic cells. At five or nine weeks of incubation the stromal layer was trypsinized, adherent cell depleted, and also assessed for CFC content.[14]

Statistics

As data related to cell and colony numbers were not normally distributed among the experiments, results are reported as median (range) unless differently stated. Comparisons were performed using the non-parametric Mann-Whitney U test. A p value ≤ 0.05 was considered to be statistically significant.

Table 1. Comparison of the production of non-adherent cells and GM-CFC during 9 weeks of culture (5 experimental pairs).

	M2-10B4 median (range)	*M2-10B4 IL3 G-CSF median (range)*
Non-adherent cells output ($\times 10^5$) (cultures of 10^6 HUCB MNC)	34 (2-370)	66.5 (5.6-580)*
Non-adherent cells output ($\times 10^5$) (cultures of 5×10^4 HUCB CD34$^+$ cells)	117 (3.9-1278)	535 (9-5882)*
GM-CFC output (cultures of 10^6 HUCB MNC)	15.5 (0-235)	9.5 (0-261)
GM-CFC output (cultures of 5×10^4 HUCB CD34$^+$ cells)	104 (0-504)	273 (0-3197)°

**p-value < 0.001 and °p-value = 0.01 compared to M2-10B4 using the Mann-Whitney U test.*

Results

Non-adherent cell output

Cells generated were almost exclusively granulocytes and macrophages. The non-adherent cell output during nine weeks of culture was about two fold higher in cultures using the transfected cell line (p value < 0.001) (Table 1 and Figure 1, panel A). Similarly, in cultures of CD34$^+$ cells on the M2-10B4 IL-3 G-CSF cell line, the total number of non-adherent cells was five fold higher than in cultures containing the parental cell line (p value < 0.001) (Table 1 and Figure 1, panel B).

Clonogenic cell output in the supernatant

Production of clonogenic cells was analyzed in all experiments and the number of clonogenic cells generated in the supernatant of the cultures compared. When cultures were initiated with mononuclear cells the number of GM-CFC generated on the M2-10B4 parental line and the transfected line were comparable (Table 1 and Figure 2, panel A). However, when CD34 cells were used, the output of GM-CFC was 2.6 fold higher in cultures on the growth factor transfected cell line (p value = 0.01) (Table 1 and Figure 2 panel B).

Effects on clonogenic cells present in the stromal layer and LTC-IC frequency

At week five and week nine the content of GM-CFC in the stromal layer from parental and transfected cell line cultures was assessed after adherent cell depletion.

In cultures inoculated with MNC, the number of GM-CFC per flask in the M2-10B4 stroma at week five was higher than in cultures in which the IL-3 G-CSF producing cell line was used. However, this was not statistically significant (mean±S.E.M = 137±118 vs. 4±3; p value=0.121) (Figure 3). In contrast, the number of GM-CFC in cultures of MNCs on M2-10B4 stroma at week 9 was more than 60 times higher than that in cultures on the transfected cell line and this was highly significant (mean±S.E.M = 86±55 vs. 1.3±1.3; p value = 0.007) (Figure 3).

In cultures initiated with the CD34$^+$ enriched cells there was again increased numbers of GM-CFC per flask at week five of culture on M2-10B4 cells compared to those in M2-10B4 IL-3 G-CSF layers. This,

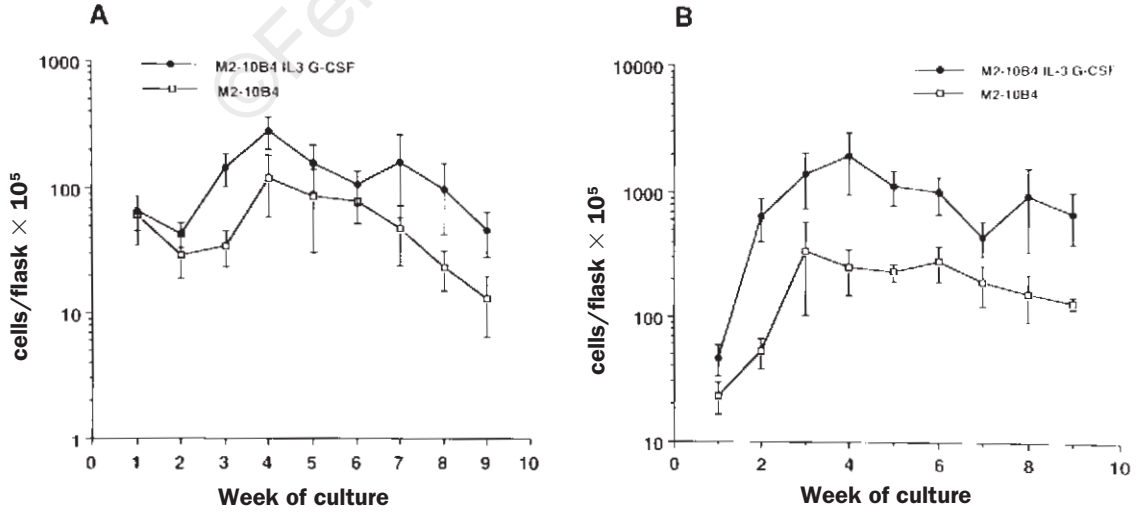

Figure 1. Non-adherent cell production per flask in long-term cultures of mononuclear cells (panel A) and CD34$^+$ cells (panel B) from CB. Results are reported as mean±S.E.M. of 5 experimental pairs.

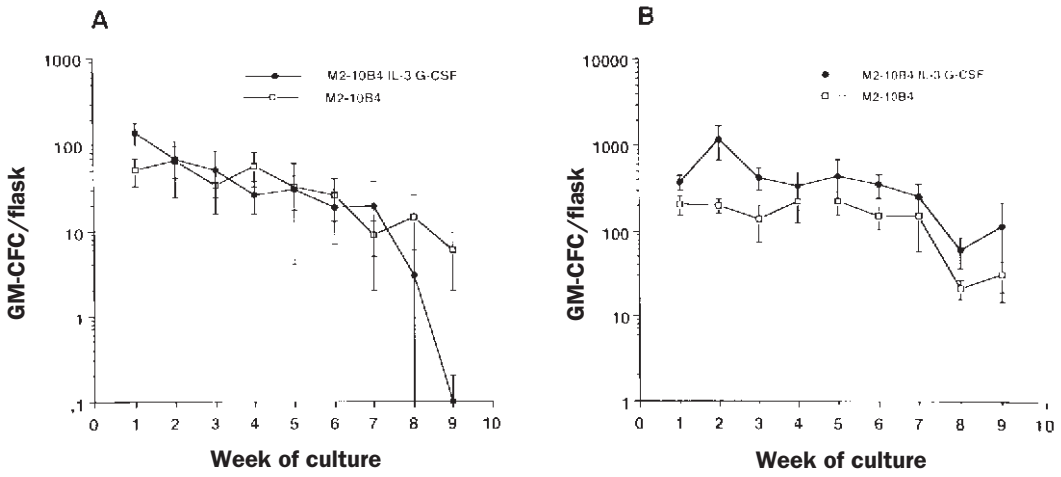

Figure 2. GM-CFC production per flask in long-term cultures of mononuclear cells (panel A) and CD34+ cells (panel B) from CB. Results are reported as mean±S.E.M. of 5 experimental pairs.

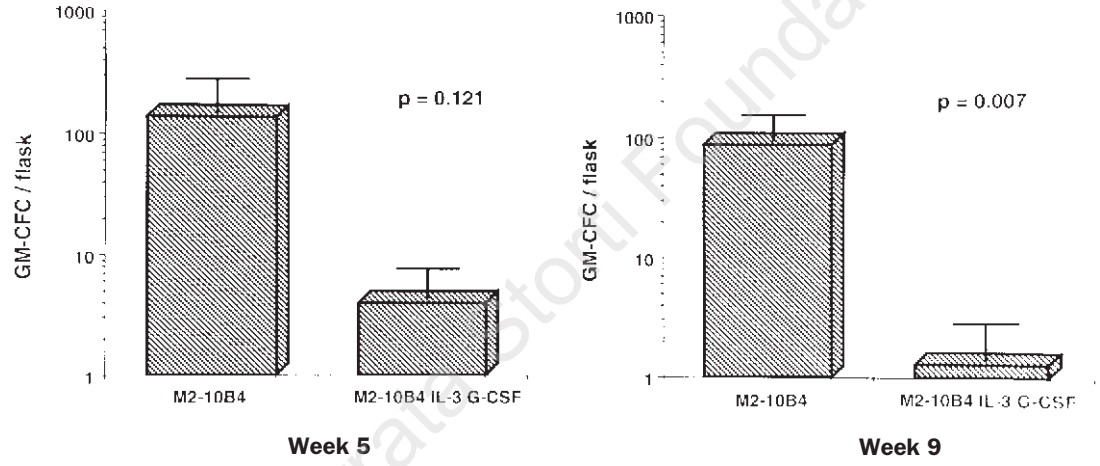

Figure 3. GM-CFC per flask present in the adherent layer of cultures of CB mononuclear cells. Results reported are means±S.E.M. and *p* values refer to the Mann-Whitney U test.

too, was statistically significant (mean±S.E.M = 701± 223 vs. 14±10; *p* value = 0.049) (Figure 4). Similarly, the number of GM-CFC per flask assayed at week 9 from cultures seeded with CD34 enriched cells was significantly higher in M2-10B4 layers, as shown in Figure 4 (mean±S.E.M = 152±52 vs. 10±10; *p* value = 0.013).

In a single experiment, aliquots of the cells harvested from the transfected and non-transfected adherent layers of 9 week old cultures previously inoculated with CD34 cells were reseeded on fresh irradiated M2-10B4 feeders. After a further 5 weeks these cultures were trypsinized and cells assessed for residual clonogenic content. No colony-forming cells were generated from cells harvested from the adherent layer of the M2-10B4 IL-3 G-CSF culture. Adherent cells harvested from the M2-10B4 culture, however, were still capable of generating GM-CFC at an estimated frequency of 1 GM-CFC per 17,307 cells reseeded on fresh stroma.

Discussion

The use of continuous cell lines, of human or murine origin, is increasingly reported and is a very useful alternative to normal human bone marrow for hematologic experimentation.[1-6] Nevertheless, we have recently demonstrated that the use of the M2-10B4 stromal cell line when compared to allogeneic bone marrow stroma can lead to different results in the estimation of LTC-IC frequency.[11] In the present study we found that the proliferative capacity of LTC-IC present in CB may be strongly influenced by the type of supportive stroma. The presence of stimula-

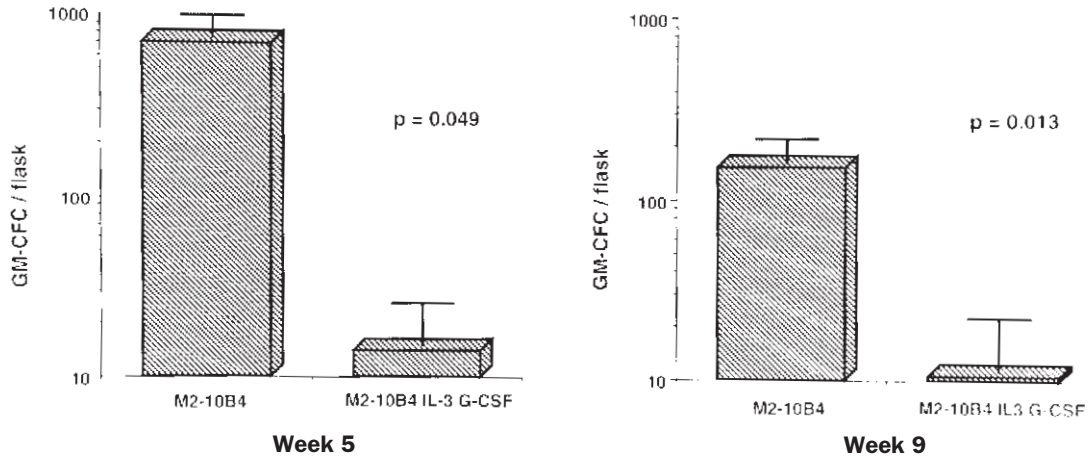

Figure 4. GM-CFC per flask present in the adherent layer of cultures of CB CD34 enriched cell population. Results reported are means±S.E.M. and *p* values refer to the Mann-Whitney U test.

tory growth factors generated by the transfected cell line (M2-10B4 IL-3 G-CSF) may thus affect the *in vitro* behavior of LTC-IC and the ability to quantify them accurately.

By co-culturing CB mononuclear cells on the two murine feeders, we observed that over a 9 week culture period, the generation of nonadherent cells was two fold higher in cultures containing the transfected cell line. In experiments initiated with the $CD34^+$ enriched cells the non-adherent cell output on the M2-10B4 transfected cultures increased to five fold higher than the cell output from cultures on the parental cell line. These results are in line with those previously reported for cultures of $CD34^+$ bone marrow subpopulations on the same supportive cell lines where the enhanced cell output was attributed to the stimulatory effect of IL-3, G-CSF or GM-CSF on granulopoiesis.[1]

Analysis of the clonogenic cell output showed that comparable numbers of progenitors were produced on both stromal cell lines when cultures were initiated with CB mononuclear cells. Better support (over two fold difference) was provided by the transfected feeders in cultures inititiated with the $CD34^+$ enriched fraction. Accessory cells present in the mononuclear fraction (and not in the $CD34^+$ enriched population) might synergize with the cytokine-producing feeder cells by producing other cytokines and by causing increased maturation of LTC-IC and therefore a reduced maintenance of the latter. An alternative possibility, which we could not rule out, might be related to an increased number of LTC-IC recruited to differentiate by the growth factor producing stromal cells or to an increased proliferative capacity of the LTC-IC originally present in the inoculum.

When CB $CD34^+$ cells were used as a starting population we found that the number of residual CFC in the stromal layers of cultures on the transfected cell line, at both week 5 and week 9, was significantly low. The opposite was true in the supernatant of the same cultures, where higher numbers of terminally differentiated cells and CFC were found in the presence of M2-10B4 IL-3 G-CSF feeders. This may simply suggest a different distribution of CFC between stroma and supernatant in the presence of the two stromal layers. An alternative explanation is that in cultures in which IL-3 and G-CSF are continuously generated, the proliferative capacity of the LTC-IC has been maximized leading to higher numbers of terminally differentiated cells and progenitors in the supernatant. This may lead to more rapid exhaustion of the LTC-IC. This interpretation is further supported by the inability of the cells harvested from the transfected feeders to generate GM-CFC in secondary cultures. This was in marked contrast to secondary cultures using the parental cell line in which cells from the adherent layer were able to generate CFC when replated on fresh stroma.

Our findings are different from those previously reported using BM derived $CD34^+$ cells, where the transfected murine stromal cell line was found to support the maintenance of LTC-IC, better than the parental cell line.[1] This may be related to the different behavior of LTC-IC from CB compared to that of LTC-IC from BM. Functional studies, besides phenotype, of $CD34^+$ cells from CB and BM provide evidence in favor of this difference showing that $CD34^+$ $CD38^-$ CB cells (enriched for LTC-IC) have a higher cloning efficiency and proliferation rate in response to cytokine stimulation than their BM counterparts.[16,17]

It was recently reported that there was a significant difference in the number of CFC produced per LTC-

IC from CB when the M2-10B4 stromal cell line or a 1:1 mixture of M2-10B4 IL-3 G-CSF cells and Sl/Sl cells (a mouse stromal line engineered to produce stem cell factor (SF) and IL-3) were compared.[7] The same study reported an increased plating efficiency of LTC-IC from normal bone marrow, CB and mobilized blood.[7] The authors concluded that cells not detectable as LTC-IC on normal bone marrow feeders were recruited in the co-cultures as these cell lines express high levels of human growth factors. In our study the effects on LTC-IC behavior are highly dependent on the stromal cell line used. The different types of feeders could influence not only the LTC-IC proliferative potential but also their plating efficiency. As the cell lines constitutively express growth factors, this would lead to continuous stimulation of the primitive LTC-IC resulting in rapid exhaustion.

The influence on CB LTC-IC exerted by the murine transfected stromal cell line is reflected by the estimation of LTC-IC present in the inoculum when proliferative capacity is considered as the end point. The assumption of a uniform proliferative capacity for LTC-IC derived from different sources (bone marrow, growth factors stimulated peripheral blood, cord blood) and assessed by different culture conditions (human normal bone marrow, human or murine stromal cell lines as feeder support) has led many authors to estimate the LTC-IC frequencies by simply calculating the CFC output after 5 weeks of culture.[9,10] If we were to assume, for the LTC-IC derived from CB, a proliferative capacity similar to the LTC-IC present in human bone marrow or normal human peripheral blood, we could estimate their frequency by adding supernatant and adherent CFC and assuming 4 CFC generated per LTC-IC.[8,18] The average incidence of week 5 LTC-IC in M2-10B4 cultures was 1:182 $CD34^+$ cells (range 1:159-1:343) in agreement with previous estimates for $CD34^+$ cells. In cultures using M2-10B4 IL-3 G-CSF cells, the average frequency of week 5 LTC-IC was 1: 1,789 $CD34^+$ cells (range 1: 806-1:4,166), over 9 fold lower than in cultures using the parental cell line. The same holds true for a calculation of LTC-IC at week 9. A 1.5 fold higher frequency was found in cultures utilizing M2-10B4 cells than in those using the transfected cells (1:626 $CD34^+$ cells, range 1:378-1:3,425 and 1:960 $CD34^+$ cells, range 1:286-1:30,150, respectively).

In this study we have shown that there can be a 9 fold difference in estimation of LTC-IC frequency when two different murine stromal cell lines are used in LTCs. This can be attributed to the different number of CFC detectable in the adherent layer of cultures at week five or week nine.

In summary our results reinforce the notion that long-term culture on stromal support other than normal bone marrow can strongly influence the *in vitro* behavior of LTC-IC and may lead to errors in estimating the frequency of LTC-IC.

Contributions and Acknowledgments

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Disclosures

Conflict of interest: none.

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Manuscript processing

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