



Progenitor cell expansion

The CD34⁺CD38^{neg} population is significantly increased in haemopoietic cell expansion cultures in serum-free compared to serum-replete conditions: dissociation of phenotype and function

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Summary:

Expansion of haemopoietic stem cells is proposed to combat graft failure in adult recipients following cord blood (CB) transplantation. Cultures are traditionally performed in medium containing FCS, but to transfer expansion to the clinic, 'good manufacturing practice' (GMP) standards are required. This study evaluated expansion cultures in culture bags and serum-free (SF) conditions, to comply with GMP, by analysing sub-populations of CD34⁺ cells, colony-forming cells (CFC) and long-term culture initiating cells (LTC-IC). CD34⁺ cell analysis has previously been used to measure clonogenic capacity and the CD34⁺CD38^{neg} surface phenotype to measure primitive cell numbers. In this study, comparison of expansion in serum-replete medium with that in SF conditions demonstrated a lack of expression of CD38 on CD34⁺ cells in the absence of serum. These findings must be considered in clinical studies using *in vitro* expansion in SF conditions, and the CD34⁺CD38^{neg} phenotype should not be used to confirm maintenance, or expansion, of primitive progenitor cells. *Bone Marrow Transplantation* (2001) 27, 365–371.

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Haemopoietic stem cells from cord blood (CB) can be transplanted to treat children with potentially fatal marrow diseases.^{1–3} The CD34⁺ population contains primitive haemopoietic cells including marrow repopulating cells which cannot be quantified *in vitro*; long-term culture initiating cells (LTC-IC) which are CD38^{neg} with high CD90 (Thy-1) expression⁴ and committed progenitors such as colony-forming cells (CFC). Committed CD34⁺ cells may co-express lineage-specific antigens, for example, CD13 and CD33 if myeloid, CD71 if erythroid, CD19 if B cell or CD61 if megakaryocytic.⁵ Results from clinical transplants show that the total nucleated cell (TNC) and CD34⁺ cell

dose infused correlate with post-transplant survival.^{6,7} Despite optimisation of collection and processing techniques this limits CB transplantation to paediatric recipients.⁸

Expansion of stem cells is a strategy to combat graft failure in adult recipients.^{9,10} Cultures are traditionally performed in flasks with medium containing FCS,^{10–12} but to transfer expansion to clinical practice, 'good manufacturing practice' (GMP) standards are required.^{13–15} Although GMP entails procedure establishment and validation, quality system documentation and maintenance, training and laboratory accreditation amongst many directives, there are two requirements in particular that may impact on the quality of the cell expansion. The first is the removal of bovine products from the processing of clinical material following the BSE crisis, and the second is the utilisation of enclosed culture in bags to produce a reliable, safe and sterile therapeutic product comparable to other blood products issued by the Transfusion Service. This change from culture in flasks to bags may be of significance to the health of the cultures because of the larger surface area available for gas exchange.

The measurement of CD34⁺ cells provides a rapid practical alternative to CFC assays^{16,17} and has been used to measure clonogenic capacity.¹⁸ Similarly, the CD34⁺CD38^{neg} surface phenotype has been used to measure primitive cell numbers^{19,20} and their expansion in culture.^{9,11} This study evaluated expansion cultures in serum-free (SF) media, which would be used under GMP conditions, in culture bags using phenotypic and functional assays to determine the number of haemopoietic cells.

Methods

Cell preparation

Cord blood was obtained from normal full term deliveries.²¹ CD34⁺ cells were isolated from the CB mononuclear cell fraction using a MiniMACS CD34 isolation kit (Miltenyi Biotec, Bisley, UK) according to the manufacturer's instructions.¹⁰

Expansion cultures

In four paired experiments, enriched CB CD34⁺ cells were seeded at 5×10^3 /ml into 25 cm² vented culture flasks (Fahrenheit, Milton Keynes, UK) and VueLife FEP culture bags (a gift from Amgen, Thousand Oaks, CA, USA). Cells were cultured in IMDM (Sigma, Poole, UK) containing 20% FCS (Stem Cell Technologies, Vancouver, Canada) or serum-free 'Modified Ex-vivo Expansion Medium' (a gift from Amgen). All cultures contained 10 ng/ml Flt-3, IL-3, IL-6 (PeproTech EC, London, UK), G-CSF, SCF and M-GDF (gifts from Amgen). On days 7 and 10, flasks and bags were fed by half medium changes and TNC, CD34⁺ and CFU-GM content assessed. It was not possible to assess the number of LTC-IC present at day 0 in this first series because of the number of cells required for the initiation of the four expansion cultures and the other parameters being measured.

Subsequent to these experiments, which showed there was no significant difference in expansion in flask or bag cultures, a second series of experiments were performed ($n = 4$), in which CD34⁺ cells were seeded only into tissue culture bags in either serum-free or -replete medium. This enabled the assessment of LTC-IC expansion in addition to the previous parameters.

Flow cytometry

Cells from expansion cultures were assessed for CD34⁺ cell content by dual labelling with FITC-conjugated anti-CD45 (DAKO, High Wycombe, UK) and with a phycoerythrin (PE)-conjugated anti-CD34 (HPCA-2 clone, Becton Dickinson (BD), Cowley, UK) on a Coulter Epics XL flow cytometer and analysed with System II software (Beckman Coulter, High Wycombe, UK). CD34⁺ cell subset analysis was performed using FITC-conjugated CD38, CD13, CD33 (DAKO), PE CD90 (BD) and Cy5-conjugated CD38 (Beckman Coulter).

Clonogenic assays

CFCs were assayed as described previously^{10,18} using 1.3% methylcellulose in IMDM containing 2 U/ml erythropoietin (Eprex; Janssen-Cilag Ltd, High Wycombe, UK), 10% 5637 conditioned medium, 10% BSA (Stem Cell Technologies) and 30% FCS. Triplicate cultures were plated in 0.25 ml volumes in 24-well tissue culture plates. CD34⁺ enriched cells were plated at 125 cells per well. After the percentage of CD34 cells in the expansion cultures had been determined, the CFC assays were seeded with appropriate cell concentrations, ranging from 1×10^4 to 1×10^6 /ml, to give 125 CD34⁺ cells per well. After 14 days at 37°C in a humidified air/5% CO₂ atmosphere, cells were scored for the presence of CFC.

The frequency of week 5 LTC-ICs was determined using a limiting dilution assay as previously described.²² Cells from expansion cultures were diluted to give six different concentrations of CD34⁺ cells, ranging from 12.5 to 400 CD34⁺ cells per well, and cultured with cryopreserved irradiated bone marrow stroma²² in 96-well plates with 24 replicates per dilution. After 5 weeks non-adherent and

adherent cells were harvested from individual wells of three chosen dilutions by the addition of 0.25% trypsin and assayed for CFC.¹⁰ LTC-IC frequencies were calculated with the Poisson formula from the percentage of negative wells using the Strijbosch computer programme.²³

Results

Expansion cultures

The expansion of TNC, CD34⁺ cells and CFC obtained in the first series is shown in Table 1. The mean fold expansion after 14 days serum-replete flask culture conditions were TNC 2475 (s.d. 468), CD34 20 (s.d. 15), CFC 14 (s.d. 11) compared to TNC 1416 (s.d. 158), CD34 79 (s.d. 20), CFC 16-fold (s.d. 7) with serum-free culture in bags ($n = 4$). This series of experiments demonstrated no significant difference between culture in flasks compared to bags, whereas culture in SF conditions in flasks or bags resulted in less TNC expansion ($P = 0.01$), but greater CD34 cell expansion ($P = 0.003$) than in the presence of serum. Consequently for the subsequent four paired expansion experiments, CD34⁺ cells were only seeded into tissue culture bags with either IMDM + FCS or SF medium.

The details of all eight cord blood samples expanded in culture bags in the presence of serum-replete or serum-free medium are given in Table 2. Overall mean fold expansion with serum was TNC 2308 (s.d. 478), CD34 19 (s.d. 8) CFC 17 (s.d. 12) and without TNC 1096 (s.d. 486), CD34 54 (s.d. 31) and CFC 18 (s.d. 7). The expansion of LTC-IC obtained after 14 days pre-culture in both conditions was comparable, the mean fold expansion with FCS was 4.9 (s.d. 0.88) and in serum-free conditions 4.4 (s.d. 0.78).

Cell surface antigen expression

Subset analysis of CD34 cells in the two culture conditions demonstrated significantly different CD38 expression (Table 3), mean CD34⁺CD38⁺ 12% (s.d. 7) in SF conditions compared to 96% (s.d. 4) in medium + FCS ($P < 0.001$, $n = 8$). There was no significant difference in the expression of CD90, approximately 6%, nor CD61, approximately 7% under both conditions (data not shown). Most CD34⁺ cells co-expressed CD13 after 7 days in culture (90.2%, s.d. 3.1 in IMDM + FCS and 98.3%, s.d. 1.3 in serum-free conditions, $n = 4$), with very little variation after 10 and 14 days. At no time did the difference between the levels of CD13 co-expression on the CD34⁺ cells under the different culture conditions reach statistical significance. There was great sample variation in the expression of CD33. In one cord blood sample, only 12.9% of CD34⁺ cells co-expressed CD33 after 7 days of culture with IMDM + FCS and 11.3% in SF conditions, while in another sample the number of CD34⁺ cells co-expressing CD33 by day 7 were 91.8% and 91.9%, respectively.

CD38 expression

The increase of the CD38^{neg} population under SF conditions, in comparison with culture in the presence of

Table 1 Comparison of expansion of TNC, CD34⁺ cells and CFC after 14 days culture under four different culture conditions

			Day 0	Day 7	Day 10	Day 14
TNC mean (s.d.) × 10 ⁴	Flask	IMDM/FCS	5 ^a	946 (280)	4395 (353)	12375 (2333)
	Flask	SF	5	475 (133)	2523 (692)	8535 (1074)
	Bag	IMDM/FCS	10	2078 (697)	9380 (2751)	21967 (6862)
	Bag	SF	10	1037 (353)	4560 (598)	14150 (1605)
CD34 mean (s.d.) × 10 ⁴	Flask	IMDM/FCS	4.6 (0.4)	73 (22)	99 (61)	93 (72)
	Flask	SF	4.6 (0.4)	86 (23)	204 (81)	425 (133)
	Bag	IMDM/FCS	9.2 (0.8)	189 (88)	271 (174)	154 (73)
	Bag	SF	9.2 (0.8)	207 (69)	446 (126)	742 (235)
CFC mean (s.d.) × 10 ⁴	Flask	IMDM/FCS	1.7 (0.9)	8 (3)	14 (9)	20 (13)
	Flask	SF	1.7 (0.9)	12 (3)	19 (9)	26 (8)
	Bag	IMDM/FCS	3.4 (1.9)	19 (13)	31 (21)	29 (13)
	Bag	SF	3.4 (1.9)	27 (11)	28 (15)	44 (9)

^aCD34⁺ cells were seeded at 5 × 10³/ml into culture flasks (10 ml) and culture bags (20 ml) (n = 4).

Table 2 Comparison of expansion of cord blood CD34⁺ cells in tissue culture bags in the presence of serum-replete or serum-free medium

	Experiment							
	1	2	3	4	5	6	7	8
<i>Day 0</i>								
TNC × 10 ⁵	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CD34 ⁺ × 10 ⁵	0.97	0.94	0.98	0.80	0.95	0.93	0.93	0.97
CFU × 10 ⁵	0.26	0.63	0.24	0.24	0.17	0.17	0.23	0.24
LTC-IC freq					1/26	1/22	1/24	1/55
LTC-IC					3846	4545	4146	1818
<i>Day 14 IMDM/FCS</i>								
TNC × 10 ⁵	Inf	1572	2088	2936	2244	2910	2180	2228
CD34 ⁺ × 10 ⁵	Inf	17.60	21.30	7.21	6.72	23.90	25.30	24.50
CFU × 10 ⁵	Inf	3.26	4.01	1.56	1.27	6.13	6.63	3.57
LTC-IC freq					1/14285	1/14706	1/10753	1/20000
LTC-IC					15681	19787	20273	11150
<i>SF medium</i>								
TNC × 10 ⁵	1608	1224	1392	1440	1492	756	468	389
CD34 ⁺ × 10 ⁵	87.60	86.90	83.30	39.00	23.10	30.20	24.00	28.20
CFU × 10 ⁵	4.94	3.10	4.82	4.61	3.66	4.70	4.06	2.70
LTC-IC freq					1/10417	1/3957	1/2710	1/3906
LTC-IC					14304	19202	17269	9959

LTC-IC freq = frequency of LTC-IC per nucleated cell. LTC-IC assessment was not performed in the first four experiments.
Inf = culture infected.

serum, was consistent in all experiments and was seen on all cells, not just the CD34⁺. This is clearly demonstrated in Figure 1 which shows the results from a representative experiment examining the co-expression of CD13, CD34 and CD38 after 7 days culture in both conditions. The CD34⁺CD38^{neg} cells apparently expanded in SF conditions were not immature as CD13 and CD33 were co-expressed (Figures 1 and 2).

The lack of expression of CD38 on CD34⁺ cells after 14 day culture in serum-free conditions led to the apparent mean fold expansion of CD34⁺CD38^{neg} cells of 489, compared to a corresponding mean fold expansion of only 10 in medium containing FCS. After 14 day expansion cultures in serum-free conditions there were sufficient cells to perform all the required assessments and retain half the cells for further tests. Cells that had been cultured in SF medium

were re-cultured in IMDM + FCS, with no additional growth factors, and their CD34/CD38 levels measured 4 hourly over a 24 h period. After 14 days in serum-free medium only 5.3% of the CD34 cells expressed CD38. This remained relatively unchanged for the first 8 h in serum-replete medium, but by 16 h 25% of the CD34⁺ cells were CD38⁺, rising to 42% after 20 h and 77% by 24 h.

Discussion

We had previously reported expansion of cord blood progenitor cells in traditional culture flasks with growth factors in medium containing FCS.¹⁰ However, expansion cultures for clinical transplantation require the adherence to GMP conditions.¹³⁻¹⁵ To ensure a reliable, safe and sterile thera-

Table 3 Analysis of CD34⁺ cells in serum-free and -replete medium demonstrating significantly different CD38 expression

	<i>Experiment</i>							
	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>
<i>Day 0</i>								
CD34 ⁺ cells × 10 ⁵	0.97	0.94	0.98	0.80	0.95	0.93	0.93	0.97
% 38 ⁺	93.49	90.38	89.41	90.78	95.08	94.5	91.35	79.71
% 38 ^{neg}	6.51	9.62	10.59	9.22	4.92	5.5	8.65	20.29
CD34 ⁺ CD38 ⁺ × 10 ⁵	0.91	0.85	0.88	0.73	0.90	0.88	0.85	0.77
CD34 ⁺ CD38 ^{neg} × 10 ⁵	0.06	0.09	0.10	0.07	0.05	0.05	0.08	0.20
<i>Day 14 IMDM/FCS</i>								
CD34 ⁺ cells × 10 ⁵	Inf	17.60	21.30	7.21	6.72	23.90	25.30	24.50
% 38 ⁺		98.23	99.67	98.18	96.05	86.9	96.36	98.97
% 38 ^{neg}		1.77	0.33	0.82	3.95	13.1	3.64	6.03
CD34 ⁺ CD38 ⁺ × 10 ⁵		17.29	21.23	7.08	6.45	20.77	24.38	24.25
CD34 ⁺ CD38 ^{neg} × 10 ⁵		0.31	0.07	0.06	0.27	3.13	0.92	1.48
<i>SF medium</i>								
CD34 ⁺ cells × 10 ⁵	87.60	86.90	83.30	39.00	23.10	30.20	24.00	28.20
% 38 ⁺	8.37	25.38	19.92	9.42	13.6	4.1	9.78	5.33
% 38 ^{neg}	91.63	74.61	80.08	90.58	86.4	95.9	90.22	97.67
CD34 ⁺ CD38 ⁺ × 10 ⁵	7.33	22.06	16.59	3.67	3.14	1.24	2.35	1.50
CD34 ⁺ CD38 ^{neg} × 10 ⁵	80.27	64.84	66.71	35.33	19.96	28.96	21.65	27.54

*%CD38⁺ and *%CD38^{neg} denote the percentage of CD34⁺ cells expressing CD38.
Inf = infected culture.

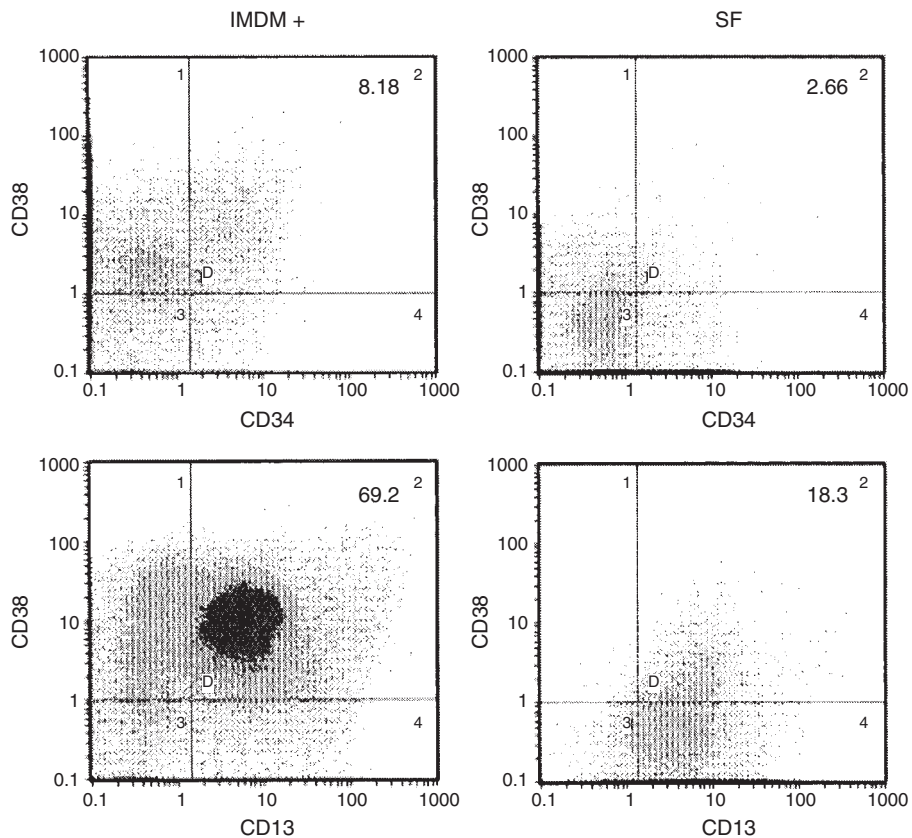


Figure 1 Lack of CD38 expression in SF culture. These flow cytometric dotplots from a representative experiment show the down-regulation of CD38 on the same cells cultured for 7 days in either IMDM/FCS or SF medium demonstrating that CD38 loss was seen on all cells, not just the CD34⁺ cells.

peutic product laboratories are utilising enclosed culture systems in bags and, subsequent to the BSE/CJD concerns, have moved away from the use of bovine serum. This study therefore set out to evaluate the expansion of LTC-IC, CFC, TNC, total CD34⁺ cells and their subsets obtained with serum-free medium in tissue culture bags in comparison to traditional flask culture.

The first series of expansion experiments were controlled with expansion cultures in flasks with serum-free medium and bags with IMDM/20% FCS (Table 1). There was no significant difference between culture in flasks compared to bags, with the larger surface area for gas exchange, whereas culture in SF conditions in either flasks or bags resulted in less TNC expansion, $P = 0.01$, but greater CD34 cell expansion, $P = 0.003$. Thus, SF conditions expanded the CD34 population and induced less maturation than traditional culture conditions. Although there was greater expansion of CFC in SF, compared to serum-replete conditions, this was not statistically significant ($P = 0.14$).

In the subsequent experiments the CD34⁺ cells were only seeded into tissue culture bags in either serum-free or -replete medium, allowing sufficient cells for the assessment of LTC-IC expansion. After 14 days in cultures containing FCS, overall mean fold expansion of CD34⁺ cells was 19 (s.d. 8) ($n = 8$) and CFC 17 (s.d. 12), but because of the greater expansion of CD34⁺ with less maturation to mature cells, the mean fold expansion obtained in serum-free conditions was 54 (s.d. 31) for CD34⁺ cells, but only

18 (s.d. 7) for the CFC. Thus the correlation between CD34⁺ cells and CFC¹⁸ held true in serum-replete culture, but not in SF medium. The expansion of LTC-IC was comparable between traditional serum-replete (4.9-fold) and serum-free conditions (4.4-fold). As there was also no difference in the expansion of CFC over all eight experiments ($P = 0.5$), the increase in expansion of CD34⁺ in SF conditions over that in serum-replete was considered due to maturing CD34⁺ cells which had lost clonogenic potential.

This conclusion was at odds with CD34 subset analysis that demonstrated significantly different CD38 expression in the two culture conditions ($P < 0.001$). In serum-replete culture 96% (s.d. 4) of CD34⁺ cells expressed CD38, resulting in 10-fold expansion of CD34⁺CD38^{neg} cells, compared to 12% (s.d. 7) in SF conditions, equivalent to an apparent 489-fold expansion of CD34⁺CD38^{neg} cells. These results indicate that while analysis of CD34⁺CD38^{neg} expression to measure primitive cell numbers¹¹ may be valid for traditional culture medium, it is not applicable to SF culture, where in comparison to four-fold LTC-IC expansion CD34⁺CD38^{neg} cells apparently expanded 489-fold.

LTC-IC are CD34⁺ cells that co-express CD90,⁴ are CD38^{lo} or CD38^{neg},^{9,20} without lineage-specific antigens. During culture CD34⁺ cells acquire CD38 and differentiate into lineage-specific progenitors.⁵ Differentiation into the myeloid lineage is characterised by acquisition of CD13 and CD33.²⁴ Phenotypic analysis indicated that only CD38

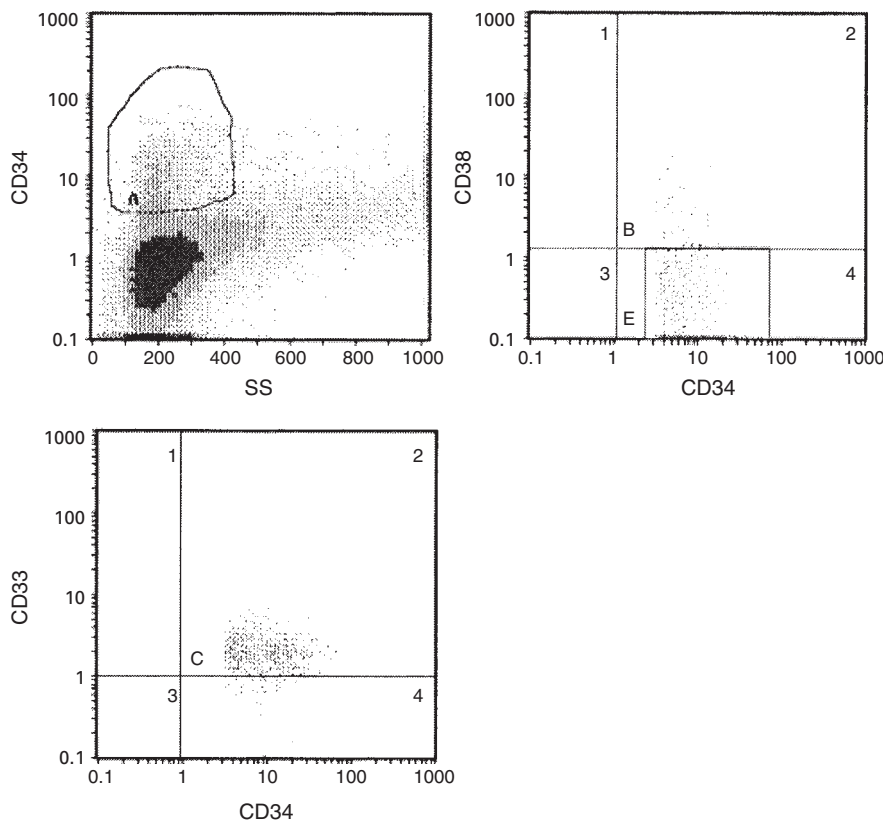


Figure 2 CD33 expression on CD34⁺CD38^{neg} cells. The same cells were examined for co-expression of CD34, CD38 and CD33. Gating first on CD34⁺ cells (Region A), then those CD34⁺ that were also CD38⁺ (Region E) demonstrated that the CD34⁺CD38^{neg} cells were also CD33⁺ and that CD38 was the only surface antigen whose expression was affected by SF culture.

expression was altered in SF culture, with no corresponding increase in expression of CD90 (5.0% with serum, 6.3% without), which would be expected if the increase in the CD38^{neg} subset was due to an expansion of the more primitive CD34⁺ cells. That the CD34⁺CD38^{neg} cells in SF conditions were not immature was confirmed by their co-expression of CD13 and CD33 (Figure 2).

Dorrell *et al*²⁵ reported the down-regulation of CD38 expression within a population of cultured CD34⁺CD38⁺ cells. They only cultured in SF conditions and concluded that the relationship between stem cell function and cell surface phenotype was not reliable for cultured cells. Our paired experiments demonstrated similar lack of CD38 in SF conditions but not in serum-replete medium, indicating the absence of FCS in the culture, not culture *per se*, to be responsible for the lack of CD38 expression. To test this hypothesis, cells that had been cultured in SF medium for 14 days were re-cultured in IMDM + FCS and their CD34/CD38 levels measured 4 hourly over a 24 h period. CD38 expression remained relatively unchanged for the first 8 h, but by 16 h 25% of the CD34⁺ cells were CD38⁺, rising to 42% after 20 h and 77% by 24 h. The rapid increase in the number of CD34⁺ cells expressing CD38 brought about simply by placing the cells in medium containing FCS, and in the absence of any growth factors, underlines the point that these cells were highly unlikely to be primitive progenitor cells.

In conclusion, direct comparison with expansion in serum-replete medium demonstrated that SF conditions interfere with CD38 expression. Because of the previous correlation between CD38 expression on CD34 cells and cell function in traditional culture systems, the CD34⁺CD38^{neg} phenotype has been used as a direct measure of primitive cell expansion.^{9,19,24} The dissociation between phenotype and function in serum-free medium reported here must be considered in clinical studies using *in vitro* GMP expansion culture, and the CD34⁺CD38^{neg} phenotype should not be used to confirm maintenance, or expansion, of primitive progenitor cells.

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