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Fibrinogen cooperates with cytokines to induce interleukin-6 receptor mRNA expression in human hematopoietic CD34+ progenitors.

Abstract

We have previously demonstrated that purified human fibrinogen (Fg), a major plasma component removed during serum preparation, shows mitogenic properties towards lymphoma cells and normal human hematopoietic progenitors. Indeed, adding Fg with IL-3 to a serum-containing medium stimulates growth of human CD34+ progenitors. In this report, we show in

serum-free medium, that this stimulating effect only occurs in the presence of IL-6. To clarify the cooperative effect between Fg and IL-6, the kinetics of IL-6 receptor (IL-6R) mRNA expression in CD34+ cells have been analyzed by semi-quantitative in situ hybridization. In the presence of both IL-3 and Fg, more cells express IL-6R mRNA, and this expression per cell is significantly greater than with each factor added separately. These results suggest that Fg does not promote the growth of normal cells by itself, but sensitizes the cells to cytokines. Fg behaves not as a “progression” factor but as a typical “competence” factor, which induces a faster and greater IL-6R expression in early human hematopoietic progenitors by cooperating with other cytokines.

Full text

INTRODUCTION

Many *in vitro* cultures of hematopoietic progenitors are performed using concentrations of cytokines higher than necessary. It has been observed that high concentrations of cytokines might have a positive effect on the growth of late progenitors, but the opposite effect on earlier stem/progenitor cells by down-regulating receptor expression on these cells [1, 2]. It is therefore important, when defining hematopoietic culture media, to combine the effects of physiological factors which can cooperate or synergize with cytokines in order to use each of them at the lowest optimal concentration. This is particularly true for studying the stem/progenitor cell compartment where tight reciprocal relations exist between the cells, the extracellular matrix scaffold and the plasma components. Thus we have shown that major plasma molecules such as fibrinogen (Fg) or purified complement C3 have mitogenic domains which bind specific growth receptors [3, 4]. These domains are different from adhesion domains such as the RGDS sequence [5, 6]. Fg is a major plasma protein (3 mg/ml) involved in multiple hematopoietic processes. It is withdrawn during clotting and the preparation of serum. Therefore serum-containing cultures normally do not contain Fg, although traces of the complete molecule or of its degradation fragments may be found in poorly prepared serum. Purified Fg, as well as its D fragment derivatives, promote a significantly more prolonged progenitor production in human long-term Dexter type cultures [3]. A 92 kDa mitogenic fibrinogen receptor (MFR), which is distinct by biological and physico-chemical criteria from known adhesion receptors such as integrins [5, 6] or ICAM antigen [7-10], has been purified and characterized. The effect of Fg added to

Fg-free serum on $CD34^+$ cells grown in mixed colony assays has been tested. Addition of 30-60 mug/ml Fg resulted in 10-fold decrease (from 1.7 U/ml to 0.17 U/ml) in the minimal concentration of IL-3 necessary to promote the development of CFU-GEMM and CFU-Mix (colony forming unit-granulocyte erythroid megacaryocyte monocyte) [11].

The multiple interactive signals that lead to the activation of the quiescent hematopoietic stem cell compartment have not yet been fully elucidated. Interleukin-6 (IL-6) has been described as one of the earliest cytokines that acts in synergy with IL-3 and G-CSF to release early progenitors from quiescence [12-14]. This implies that the IL-6 receptor (IL-6R) is expressed very early on these cells. This receptor is composed of one 80 kD subunit (alpha-chain) and one 130 kD subunit (beta-chain) and belongs to the cytokine receptor family [15]. The alpha-chain binds IL-6 and then combines with the beta-chain which promotes a transduction signal [16, 17]. The alpha-chain is specific for the IL-6R whereas the beta-chain is common to other cytokine receptors such as the CNTF, LIF and IL-11 receptors [18].

In the present study, we have observed in serum-free culture conditions with IL-3, that Fg increases the proliferation of progenitor cells when IL-6 is added. Consequently, we investigated how IL-3 and Fg cooperate with IL-6 to trigger cell proliferation. To elucidate this mechanism, we have studied, by *in situ* hybridization, the kinetics of IL-6R mRNA expression in $CD34^+$ cells cultured with a low concentration of IL-3 (0.17 U/ml) in the presence or in absence of 30 mug/ml Fg. We demonstrate here that IL-3 and Fg cooperate to increase IL-6R mRNA expression in human $CD34^+$ hematopoietic progenitors.

MATERIALS AND METHODS

Growth factors

Recombinant human (rh) IL-3 and IL-6 with the respective specific activities of 2.3×10^6 U/mg and 4×10^6 U/mg were generously provided by Genetics Institute (Cambridge, MA, USA). Rh erythropoietin (Epo) was supplied by Valbiotech and was used at 1.5 U/ml. Human Fg was purified according to Kekwick *et al.* [19]. Its purity was estimated as more than 99% by sodium dodecylsulfate polyacrylamide gel electrophoresis [3]. The purified Fg in 0.3 M NaCl can be stored for one year at - 70° C without measurable loss of biological activity.

Bone marrow samples

Specimens of bone marrow from the iliac crest, ribs or sternum were obtained either from normal bones after orthopedic surgery or from normal bone marrow transplant donors with their informed consent and under a protocol approved by the Institution's ethical guidelines. All samples were collected on heparin and cells were resuspended in Iscove modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS) (Sigma). Traces of Fg or Fg D fragments were estimated to be less than 1 mug/ml by Western blot analysis.

Purification and culture of the cells

$CD34^+$ cells were obtained as previously described [20]. For proliferation experiments, $CD34^+$ cells were resuspended in PBS/BSA (0.2%), incubated with an anti-CD34 fluorescein isothiocyanate (FITC-conjugated monoclonal antibody (MoAb) 8G12 clone, Becton-Dickinson) for 30 min at 4° C and washed twice. Using a Vantage Becton-Dickinson sorter, the cells were seeded in 96-well plates (150 cells/well in 100 mul) in serum-free medium (SB A, Novastem, France) with 1.5 U/ml Epo. This medium constituted the control medium (referred to later as CM), to which low doses of IL-3 (0.17 U/ml) and/or IL-6 (1 U/ml) were added in the presence (30 mug/ml) or in the absence of Fg, as specified. After 48 hours, single cells and doublets were counted under a Leitz inverted microscope.

For *in situ* hybridization (ISH) experiments, cells from the same preparation were plated at 2×10^5 cells/ml in 96-well tissue culture plates (Falcon) under the same culture conditions. Cells were incubated at 37° C in a humidified atmosphere containing 5% CO_2. At 12, 40, 96 and 144 hours, they were harvested and then processed for ISH.

Methylcellulose colony assays were performed in serum-containing medium (SB 1d, Novastem, France). Four hundred cells/ml were plated and incubated for 14 days. Colonies were then counted and scored under an inverted microscope, according to criteria already described [11].

In situ *hybridization (ISH)*

The complete procedure is based on a modifed method described by Angerer *et al.* [21] and Bernaudin *et al.* [22] and has been detailed in a previous report [1].

The IL-6R probe (corresponding to the alpha-chain) was a generous gift from Dr. T. Hirano [23] (Osaka, Japan). It is a 2.2 kb human IL-6R Hind III/XbaI cDNA insert present in the pIBI 76 vector. Antisense RNA was obtained by Sp6 RNA polymerization after linearization with Hind III. Sense RNA was obtained by T7 RNA polymerization after linearization with XbaI. For the positive controls, the glyceraldehyde phosphate dehydrogenase (GAPDH) probe (a generous gift from Dr. P. Haddad, Paris, France) was used. The labelled probes were synthesized by Riboprobe Gemini II Core System (Promega, Madison, WI) with ^{35}S-UTP (Amersham, 1,000 Ci/mmol) as specified by the manufacturer. Synthesized mRNAs were purified by phenol extraction and ethanol precipitation after alkaline hydrolysis [24]. The specific activities of the probes were usually about 1.3 x 10^9 cpm/mug. One million cpm were applied per slide. Non-specific binding was prevented by the addition of a sufficient concentration of *E. coli* tRNA (500 mug/ml). After 10 days of exposure, slides were developed and stained with Giemsa. Grains were counted using a program adapted for autoradiography on a SAMBA Technologies image analyzer (Meylan, France). In the negative controls, cells contained less than 3 ± 3 grains per cell. Consequently, on the reaction slide, each cell containing more than 10 grains was scored as a "positive cell".

Statistical analysis

The Student's t test was applied, when specified, to determine the statistical significance of the pooled data from several (n) experiments. Probabilities are detailed in the results.

RESULTS

Cooperative effect of Fg with IL-6 on the proliferation of CD34$^+$ cells

CD34$^+$ cells were plated at 150 cells/100 mul in a serum-free liquid medium containing 1.5 U/ml Epo alone (control medium referred to as CM) or various combinations of Fg (30 mug/ml), IL-3 (0.17 U/ml) and/or IL-6 (1 U/ml). Single cells and doublets were counted at 48 hours after the start of culture, to evaluate the percentage of cells triggered to divide after exposure to the different combinations of the above mentioned factors. Results of 3 independent experiments are shown in Table 1. In CM [1] or CM supplemented only with Fg [2] or IL-6 [3], the percentage of doublets was respectively 0.34 ± 0.31, 1.70 ± 1.02 and 0.74 ± 0.64. When the medium was supplemented with IL-3 [4], this

percentage increased to 5.24 ± 2.47, but further addition to IL-3 of Fg [5] or IL-6 [6] separately had no significant additional effect (respectively 4.69 ± 2.21 and 5.77 ± 2.06). Addition of Fg to IL-6 [7] gave results within the same range (4.77 ± 2.57). Conversely, when both Fg and IL-6 were added together to IL-3 [8], a twofold increase was observed (9.37 ± 1.42), as compared to IL-3 with Fg or IL-3 with IL-6 ($p = 0.01$).

The cooperative effect of Fg with IL-3 was also tested in the methyl cellulose serum-containing assay. Results are shown in Table 2. When IL-3 and Fg were added together, the total number of colonies was much higher than when only one factor was present. The cooperation between IL-3 and Fg led to a significant increase in the colony number.

Effect of Fg on IL-6R mRNA kinetics in $CD34^+$ cells

Kinetic studies were performed to evaluate IL-6R mRNA expression in $CD34^+$ progenitors. Cells from the same preparation but different from those used for proliferation experiments were cultured in CM or in CM supplemented with 0.17 U/ml IL-3 or 30 mug/ml Fg or both. They were harvested 12, 36, 96 and 144 hours after the start of culture and hybridized with an IL-6 mRNA probe. Results of the first 4 harvesting times are shown in Figure and correspond to means of 4 independent experiments. Twelve hours after the onset of the culture, when IL-3 and Fg were added together, 42% of the cells were positive with the IL-6R mRNA probe. Conversely, only 5% of the cells were positive when cultured in CM alone or supplemented with IL-3 and only 9% in CM supplemented with Fg. Various other IL-3 concentrations have been tested, but the percentage of positive cells at that time was always lower than that observed in the presence of 0.17 U/ml IL-3 with Fg (results not shown). After 36 hours of culture, an increase in IL-6R mRNA expression was observed with IL-3 or Fg alone, but it did not reach the level obtained after 12 hours with IL-3 and Fg (respectively 32 and 19%). After 4 days, the IL-6R mRNA expression was low in all culture conditions as well as after 6 days (results not shown).

In view of the peak of IL-6 mRNA expression obtained with IL-3 and Fg at 12 hours, we completed our study with an analysis of the hybridized population at that specific time. Cells were classified according to the number of grains counted per cell as shown in Table 3. The maximum number of labelled cells and grains per cell was obtained in the presence of IL-3 and Fg. Indeed in this condition,

46.1% of positively labelled cells were detected, whereas only 7% and 11% of positive cells were detected respectively in CM or in CM supplemented with IL-3 only. Among the 46.1% positive cells found in the cell population cultured with IL-3 and Fg, 26.2% were labelled 10-30 grains, 7% with 30-50 grains and 12.9% with 50-90 grains, a category which was completely absent in the other culture conditions. Indeed, in CM with IL-3 only, among the 11% positive cells, 7.6% showed 10 to 30 labelled grains and 3.4% 30 to 50 grains. No cells were observed in the 50 to 90 grain category. All the positive cells obtained in CM (7%) showed fewer than 30 labelled grains per cell.

DISCUSSION

In the present report, we show that IL-6 promotes the proliferation of human hematopoietic $CD34^+$ progenitors much more efficiently in the presence of Fg than with IL-3 alone, whereas Fg by itself does not show any significant mitogenic effect. By ISH, we show that cells exhibit an early and dramatic increase in IL-6R mRNA expression when cultured with IL-3 and Fg in association, but not with either IL-3 or Fg alone. These results suggest that Fg behaves as a "competence factor". This concept was first introduced in 1978 by Pledger *et al.*, who showed that an ordered sequence of events was required before BALB/c-3T3 becomes committed to DNA synthesis [25]. Later, these authors established that PDGF is the competence factor which conditions the cells to respond optimally to somatomedin C [26]. Here, we have observed that Fg appears to trigger hematopoietic cells to express cytokine receptors, sensitizing them to the corresponding cytokine. The proposed role as a "competence factor" for Fg is strengthened by our results obtained in semi-solid methylcellulose serum-containing assay. In addition, these results strongly suggest that the serum added to the methylcellulose contains IL-6, which triggers the cells to proliferate because Fg has rendered the cells competent to respond to this cytokine.

It is important to note that the cooperative effects observed in the proliferation experiments were obtained with very low concentrations of cytokines (IL-3: 0.17 U/ml; IL-6: 1 U/ml). When a higher concentration of IL-6 was used (2 to 4 U/ml), the synergistic effect with Fg was no longer observable (results not shown). The concentration units of IL-3 and IL-6 used in this study correspond respectively to 73 pg/ml and 125 pg/ml. In comparison to the concentrations found in the serum - respectively 0.5-15.5 and 0.4-10.1 pg/ml - these values are closer to the

physiological values than the 100-fold higher concentrations used in many other studies. We have established that cytokine concentrations are crucial for receptor expression on the cells. Indeed, we have shown that immature cells respond to lower cytokine concentrations [11] and express lower levels of receptor mRNA than more mature cells [1]. We have further shown that c-Fms (CSF-1R) mRNA is down-modulated by high concentrations of IL-3 or GM-CSF and that down-modulation is faster in normal immature cells than in mature or transformed cells [2]. It is worth-while emphasizing that the cooperative effect of Fg with IL-3 allowed us to demonstrate *in vitro* the effect of very low physiological concentrations of cytokines. Such cooperation might very well be a phenomenon which exists *in vivo*, but it is not possible to test this hypothesis directly as Fg is an important component permanently present in the plasma. In fact, our results are highly relevant for *ex vivo* expansion: indeed, as pointed out earlier, high cytokine concentrations down-modulate mitogenic receptors more rapidly on immature cells than on mature ones [2]. So, high cytokine concentrations favor the expansion of more mature progenitors rather than immature ones. Consequently, to expand cells *ex vivo* for cell therapy, it is worth working with cytokine concentrations as close as possible to the physiological ones.

A 92 Kd specific mitogenic receptor, the mitogenic fibrinogen receptor (MFR), has been isolated from established cell lines and was shown not to belong to the integrin receptor family [4, 7]. The existence of this receptor on $CD34^+$ umbilical cord blood cells has since been strongly suggested by the use of a specific antiserum which blocks the mitogenic effect of Fg observed on the same cells [10]. Our results confirm that various components of the ECM have growth factor activity [27-28]. Among these components, Fg may well be one co-activator of hematopoietic progenitor proliferation as shown by the present study.

CONCLUSION

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Figures



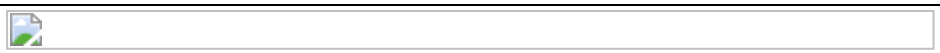


Table 1
Cooperative effect of Fg and IL-6 with IL-3 on the proliferation of CD34⁺ cells in liquid cultures

Factors added CM* (condition 1)	Exp. 1	Exp. 2	Exp. 3	Mean (± SD)	*P (between conditions)*
None (1)	0.62	0	0.4	0.34 (± 0.31)	–
F_{δ} (2)	2.81	0.8	1.5	1.7 (± 1.02)	–
IL-6 (3)	1.03	0	1.2	0.74 (± 0.64)	–
IL-3 (4)	2.54	7.4	5.8	5.24 (± 2.47)	–
IL-3 + F_{δ} (5)	3.87	7.2	3	4.69 (± 2.21)	*0.34 (4-5)*
IL-3 + IL-6 (6)	3.53	6.2	7.6	5.77 (± 2.06)	*0.34 (5-6)*
IL-6 + F_{δ} (7)	1.8	6.2	6.3	4.77 (± 2.57)	*0.97 (5-7)*
IL-3 + IL-6 + F_{δ} (8)	8.33	8.8	11	9.37 (± 1.42)	*0.01 (6-8)*

Results represent the ratio of doublets *versus* single cells at 48 hours of culture in the specified conditions. Cells were seeded at 150 cells in 100 mul.
* CM : Control medium constituted by SB A serum-free medium supplemented with 1.5 U/ml Epo.

Table 2
Mixed colonies obtained in methylcellulose assay in control medium (CM) with or without IL-3 and/or Fg

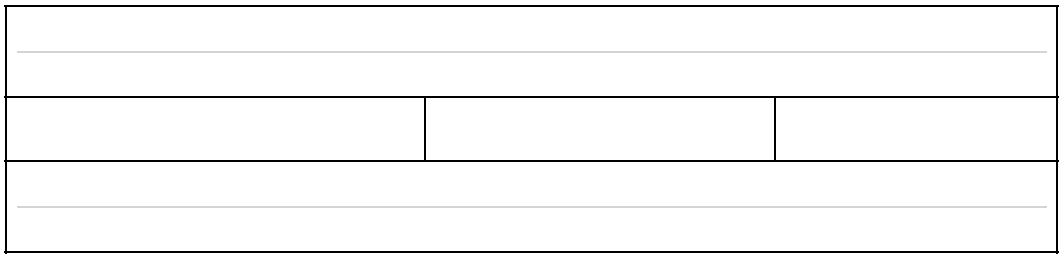
	Condition (1) CM	Condition (2) CM + F_{δ}	Condition (3) CM + IL-3	Condition (4) CM + IL-3 + F_{δ}
Exp. 1	0	1	2	4
Exp. 2	0	0	4	6
Exp. 3	1	0	3	10
Exp. 4	0	0	3	9
Total colony number	1	1	12	29
P (between conditions)		1 (1-2)	0.048 (3-4)	0.011 (1-4)

Cells were plated at 400 cells/ml in SB 1d and colonies were counted after 14 days.

Table 3
Distribution of the cells according to the number of grains per cell after *in situ* hybridization with an IL-6-R alpha chain antisense riprobe

Culture conditions	% Cells in each category: 10-30 grains	30-50 grains	50-90 grains	Total positive cells (%)
CM	7	0	0	7
CM + IL-3	7.6	3.4	0	11
CM + IL-3 + Fg	26.2	7	12.9	46.1

CD34+ cells were cultured with 1.5 U/ml Epo alone (CM), in CM with 0.17 U/ml IL-3 and in CM with IL-3 and 30 mug/ml FG. Negative cells were labelled by fewer than 10 grains. These results of one representative experiment obtained 12 hours after the onset of culture are expressed as a % of the total number of labelled and non-labelled cells. The results of 4 independent experimentss (mean ± SD) showed the following percentages of total IL-6-R mRNA expressing cells: 5% ± 1.6 in CM, 14% ± 1.4 in CM + IL-3 and 42% ± 7 in CM + IL-3 + Fg.



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human, IFN-g, IFN-g receptor, signal transduction, cellular proliferation, apoptosis.

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interleukin-6, embryo, blastocyst, endometrium, heart, gp130.

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Z. Zidek
cyclic AMP, prostaglandins, nitric oxide, interleukin-10, tumor necrosis factor-a, interferon-g.

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R.W. Freeburn, H. Kendall, L. Dobson, J. Egan, N.J. Simler, A.B. Millar
TNF-a, idiopathic pulmonary fibrosis, polymorphism.

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neuroblastoma, immunotherapy, gene therapy, cytokine, IL-1b, TNF-a.

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colorectal cancer, tumour-infiltrating lymphocytes, cytokines, gene expression, RT-PCR.

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intestinal mucosa, Crohn's disease, interleukin-18, caspase-1/ICE, immunohistochemistry, reverse transcription polymerase chain reaction.

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TNF, cytokine, LPS.

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apoptosis, caspases, Hsp27, Hsp70, T lymphocytes.

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MHC class II antigens, murine macrophages, c-Fos.

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MRC5, fibroblasts, TNF receptor, RT-PCR, binding, flow cytometry.

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IL-10, recombinant, rat, cytokines.

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