Original article

Dual Signaling of the Fas Receptor: on Saudi Patients with Chronic Leukemia.

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Abstract:

Introduction: Purifed CD34+ cells and cultured colony forming unit-granulocyte and macrophage (CFU-GM) from human bone marrow were utilized to examine the role of Fas/FasL and caspase-8interactionsin the regulation of myelopoiesis. ***Methods:*** Fas and FasL expression in CD34+ cells and in day 14 CFU-GM were measured by RT-PCR and immunofluorescence in each case. The functional tests for the CFU-GM were the standard settlement test and the proliferative limit of CFU-GM was measured by replating the essential cells and monitoring auxiliary colony development. ***Results:*** Treatment of CFU-GM with IETD significantly increased, the proliferative limit, while the Fas mAb resulted in decreased the Fas and FasL expression were measuredutilizing RT-PCR and immunofluorescence individually. ***Conclusion:*** Fas, FasL, and caspase attenuationplay important rolesin the control of myelopoiesis.

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Introduction:

Strategies aimed at the control of apoptosis in hemopoiesis might represent a mechanism to control stem and progenitor cell numberss. It would be also be feasible for dysregulation of apoptosis to promote the development of leukemia. The interaction between FasR/CD95 and with the ligand FasL/CD95L represents of the most comprehensively described initiation mechanisms for initiating the intracellular events that control apoptotis is in diverse cells types[1-2]. FasR (CD95) is a 48 kDa cysteine-rich type I cell tissue protein, and is a member of the TNF/NGF receptor family which also includes TNFR, DR3, DR4, DR5, DR6 and APRIL. FasR is comprehensively expressed by the majority of nucleated cells found in liver, lung, digestive tract, heart, kidney, ovary, different lymphomas and leukemic cells[3]. Interestingly, the expression of Fas ligand (FasL) has a much more limited expression being confined to executioner (NK) cells, cytotoxic T lymphocytes (CTL) [4]. FasR ligation leads to activation of caspases [5].

Peschle etal [6] demonstrated the critical role of Fas and FasL in controlling erythropoiesis using immunohistochemistry in normal bone marrow samples. Traver et al[7] demonstrated that granulopoiesis could be regulated comparably by a negative input component through interactions between Fas-expressing clonogenic cells and neutrophils that express FasL. We therefore

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investigated the role that attenuation of Fas, FasL, or caspase-8 play in the control of myelopoiesis

Materials and methods:

Sources of cells

Normal bone marrow (NBM) samples were acquired from subjects donating cells for allogeneic transplantation. Written informed assent and Research Ethics Committee approval were acquired in all cases. Mononuclear cells (MNCs) were gotten by a thickness angle centrifugation over Ficoll-Hypaque (1.077 g/ml) (Nyegaard, Oslo, Norway). In the wake of washing with HBSS (Mononuclear cells (MNCs) were purified by denisity gradient centrifugation over Ficoll-Hypaque (1.077 g/ml Nyegaard, washed with HBSS (GibcoBRL) and, cells resuspended in the same medium and adjusted to a concentration of 10^6/5 ml in MEM containing 15% FCS . Cell suspensions were incubated in tissue culture flasks for 2 hours at 37 º C in a humidified atmosphere with 5% $CO_2$and non-adherent cells used for culture were reaped for culture. This study was conducted between Jun 2015-Aug2016 at the Leukemia Research Unit, PSAU, Al-Kharj, Saudi Arabia.

Reagents

Recombinant human granulocyte-colony stimulating factor (G-CSF; 100 ng/ml), granulocyte-macrophage-colony stimulating factor(GM-CSF; 10 ng/ml), interleukin-3 (IL-3; 25 ng/ml) and stem cell factor (SCF;100 ng/ml) were sourced from First Link (West Midlands, United Kingdom) and optical combinations established in preliminary experiments.

IETD (Bachem, United Kingdom) was used at 10M anti-Fas Mab CH-11 was obtained from Upstate Biotechnology (Lake Placid, New York); anti-FasL Mab (65321A) was acquired from PharMingen (San Diego, California, United States) with sFasL from Alexis (San Diego, California, United States); the blocking anti-Fas Mab ZB4 utilized at 1 ng/ml was from (MBL (Nagoya, Japan); and a irrelevantIgG1 Mab (PharMingen) served as an isotype-matched control.

Clonogenic CFU-GM measure

Non-Adherent MNCs were blended with 3 ml of methylcellulose (MC) (1 x 105 cells/ml) (StemCell Technologies, Grenoble, France) containing 10 % FCS and supplemented with recombinant human cytokines (IL-3 [5 ng/ml] and SCF [20 ng/ml]). 1 ml aliquots were plated out into three 35 cm^2petri-dishesand cultured at 37 º C in humidified atmosphere with 5% CO_2 for 7 days. Colonies comprising of 50 cells or more were scored microscopically by a blinded investigator with results are expressed as the quantity of CFU-GM per 10^5 cells.

Purification of humanCD34$^+$cells

Human CD34+ cells were isolated utilizing MiniMACS immuno-magnetic beads according to the manusfacturer's instructions (MiltenyBiotec, Auburn, California). Isolated CD34+ cells were 85–98% pure as confirmed by immunostaining and flow cytometry

CFU-GM replating measure

After 7 days of culture, 120 individual colonies comprising of 50 cells or more were removed from MC utilizing a sterile Eppendorf pipette and dispersed into wells of a 96 well plate, each containing 100µl of MC in addition to FCS supplemented with IL-3 (5 ng/ml), G-CSF (100ng/ml), GM-CSF (1ng/ml), and SCF (20ng/ml). Plates were culturedfor a further 7 days at 37C in humidified atmosphere with 5 % CO_2. Colonies comprising of 50 cells or more were scored microscopically by a blinded investigator.

Results were expressed as the total extent of CFU-GM dependent colonies. Each axis shows medians plotted on logarithmic scales permitting the Area-Under-the-Curve (AUC) to be measured utilizing the Trapezium Rule. as data were skewed rather than being normally distributed. The AUC permits a general measure of colony forming cells.

RT-PCR

PCR:

Total RNA was isolated from CD34+ cells- CFU-GM at day 7 of culture utilizing a Qiagen gel cleansing pack according to the manusfacturer's instructions. The first-strand cDNA blend and PCR were performed. For the enhancement, PCR preliminaries were utilized as follows;.

Fas R: 5'- CAA GTC CAA CTC AAG GTC CAT GCC-3' (bases no. 517-540).

Fas F: 5'- CAG AGA GAG CTC AGA TAC GTT GAC-3' (bases no. 839-862).

Thirty-five cycles of PCR, were performed on a modified temperature block Hybais

Results:

We first established if Fas is expression was present in all CD34+ cells-from day 7 CFU-GM colonies and was measured by RT-PCR **(Figure 1).**

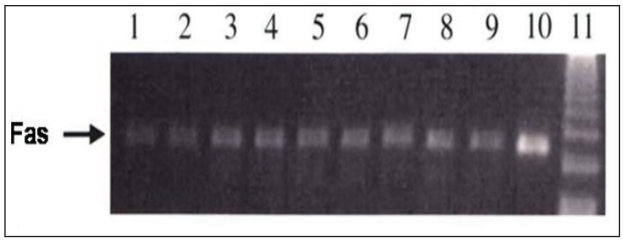

Figure 1: Expression of Fas into CD34+-cells-derived CFU-GM was determined by RT-PCR. Ten day 7 CFU-GM colonies were collected and tested by PCR. Ten colonies were PCR-positive.

cells using immunostaining. A round 90 % of day 14 CFU-GM were FasL positive (Figure 2) compared with control samples (FITC-IgG) (data not shown).

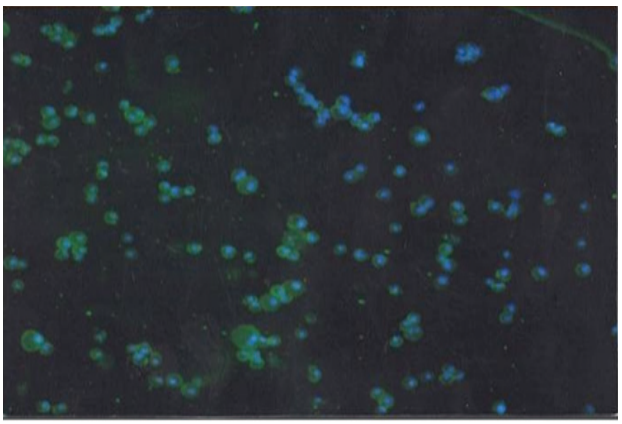

Figure 2: Immunofluorescence staining showing the FasL expression on the day 14 CFU-GM, when stained with anti-FasL FITC Mab, compared to cells stained with isotype control (not shown). Data is a representative of four experiment

Fas Mab CH-11 significantly builds the quantity of CFU-GM settlements created from HSC in liquid culture in a dosage subordinate way over a scope of 1 to 10 µg/ml. To evaluate the impact of Mab CH-11 on cell demise, 10^6 Jurkat T cells were incubated with anti-Fas Mab CH-11 at 1 µg/ml and 0.1 µg/ml. The rate of apoptotic cells was figured utilizing the trypan blue color prohibition test (> 40 % in 3 days). As a positive control, the impacts of 1 µg/ml anti-Fas CH-11 Mab on Jurkat cells were inspected. This outcome distinguished CH-11 as a viable expert apoptotic reagent in the Jurkat cell line model (information not shown). At the point when CH-11 was added to clonogenic societies in methylcellulose, there was a pattern impact on the CFU-GM plating proficiency at 10 µg/ml **(Figure 3).**

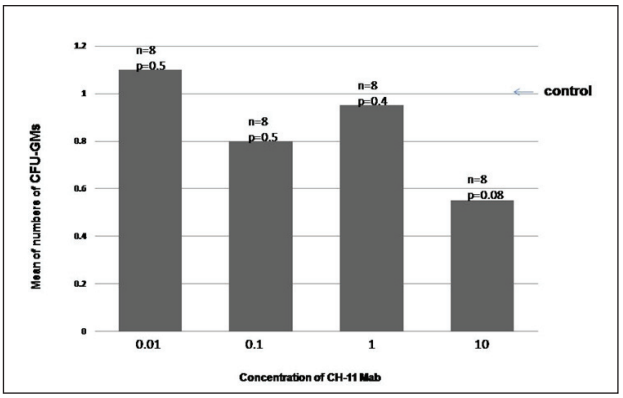

Figure 3: Number of CFU-GM colonies grown in the presence of anti-Fas Mab. There is a no significant difference between the controls and anti-Fas Mab containing plates (Wilcoxon Signed-rank test). Results are expressed as percentage of the control.

In this way, CH-11 does not seem to have any critical cytotoxic or stimulatory consequences for essential settlement arrangement when the cells are plated into methylcellulose straight away. Moreover, the outcomes in Table 1 demonstrates that there are contrast with respect to the arrangement, i.e., the cell number of day 7 and day 14 states, amongst treated and non-treated cells within the sight of rising applicationsof anti-Fas Mab.

We then explored the impact of CH-11 on CFU-GM proliferation. At 10 µg/ml, CH-11 decreased the replicating capacity (AUC) by 30% to 0.71 ± 0.1 (mean ± sem) of the control values. Neither 1 µg/ml nor 0.1µg/ml had any significant impact. The lower measurement of anti-Fas Mab 0.01 g/ml, significantly increased the replicating capacity (AUC) (p=0.02) **(Figure 4).**

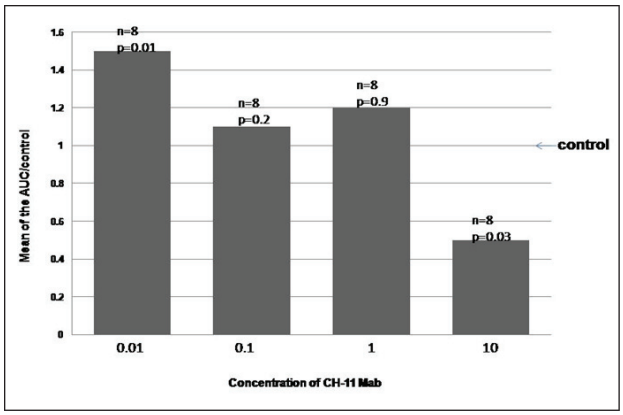

Figure 4: Self-renewal capacity (AUC) of CH-11 treated colonies. There is a significant increase in the AUC at 0.01 g/ml and a significant decrease at 10 g/ml compared to those of controls (Wilcoxon Signed-rank test).

Experiments were also performed to show that FasL was expressed by day 14 CFU-GM This outcome suggests that CH-11 may have a biphasic impact on progenitor cell multiplication. It is relevant that no enhancement of CFU-GM colony formation was seen at 10 μg/ml **(Figure 4).**

Incubation period	CH-11 Mab 10 μg/ml	CH-11 Mab 1 μg/ml	CH-11 Mab 1.1 μg/ml	CH-11 Mab 1.1 μg/ml	Control
Number of CFU-GM at 7 day (m±se)	281±2	270±5	238±4	249±2	274±3
Number of CFU-GM at 14 days (m±se)	660±2	940±7	1211±3	874±6	942±3
Frequency and Significance	n=10 p=0.05	n=11 p=0.04	n=9 p=0.02	n=8 p=0.02	n=8 p=0.05

Table 1: Number of CFU-GM colony-forming cells in methylcellulose cultures containing CH-11 at escalating concentrations. CFU-GM were assayed prior to and after 7 and 14 days of culture. There was a dramatic elevation in numbers of CFU-GM after 14 days exposure to 10μg/ml, 1μg/ml, 0.1μg/ml, and 0.01μg/ml, CH-11 Mab compared to numbers of CFU-GM at day 7.

Discussion:

The control of hemopoietic stem and progenitor cell numbers involves several elements including expansion, differentiation, and apoptosis[8]. In this manner, two primary systems may represent an expansion in hemopoietic stem/ancestor cell numbers. Initially, a decrease in projenitor cell numbers by apoptosis may have significant effects on hemopoiesis [9]. Secondly, an expansion in progenitor cell multiplication may be to the detriment of differentiation. Until recently, studies on the involvement of Fas and FasL in hemopoiesis have concentrated on their pro-apoptotic capacities [10-11]. We assessed the impact of the Fas and FasL pro-apoptotic pathway on myeloid progenitors in primary and auxiliary CFU-GM acquired from normal human bone marrow. We examined the hypothesis that Fas/FasL and downstream caspase involvement assume roles in controlling myeloid progenitor expansion and separation.

The ani Fas Mab CH-11 has been utilized by two other groups who demonstrated that it stimulated human hemopoietic progenitor cell proliferation despite the fact that it is widely acknowledged that Fas ligation is usually associated with strongly inducing apoptosis in Fas-expressing cells. Ligation of Fas with CH-11 Mab clearly diminished the AUC, demonstrating that Fas expression was connected with the functional inhibition of CFU-GM. Thus, we propose that the Fas/FasL framework is to some extent involved in the inhibition of myelopoiesis in human CFU-GM. Essential CFU-GM(s) were generated in the presence of anti-Fas Mab. anti-Fas Mab had no impact on essential CFU-GM. but it had a biphasic impact on replating. Enhanced proliferation of progenitor cells was seen at the lowest concentration of 0.01μg/ml anti-Fas Mab in methylcellulose culture. It is conceivable, that anti-Fas Mab inhibits expansion at higher concentrations, presumably because of a capacity to imitate downstream apoptotic signals. Interestingly, at lower concentrations, it might permit access of FasL to Fas and lead to a proliferative impact. In contrast, the observed 1.6-fold increment was lower than the 2.5-fold increment observed in wild type mouse culture [6-7]. This outcome indicates that apoptosis may assume a part in regulating hemopoietic progenitor cell function in humans as observed in mouse models, supporting a role for caspase activation in myeloid development.

Additionally, since Fas is a multifunctional protein placed within the TNF super-family, it can trigger and actuate two separate pathways. Fas binds to FADD death domains, prompting recruitment of caspase-8, which in turn promotes apoptosis. It should be pointed out that the 0.01g/ml concentration is much lower than the smallest concentration utilized by Barcena et al when they demonstrated a proliferative response in the presence CH-11. Despite the fact that the precise role of NFκB in apoptosis is not completely understood, two studies have demonstrated that by

NFκB initiation can inhibit apoptosis in neurons [12] and non-neuronal cells [13]. Our findings are contradicited by Kim et al [14] who found that CD34+ cells do not express Fas and that they are resistant to anti-Fas Mab and sFasL-induced apoptosis. In addition, they demonstrated that CD34+ cells express FLIP which protects cells from Fas-dependent apoptosis. Upon GM-CSF withdrawal or Fas actuation, caspase action is increased by means of either the caspase-9 pathway or the caspase-8 pathway, respectively, which results finally in apoptosis. Along these lines, we have described another capacity of caspases as controllers of myeloid expansion. In conclusion our findings support an important role for the Fas/FasL pathway and caspase initiation in inhibition of cell expansion and promotion of differentiation, supporting a role for caspases in non apoptotic functions in the control of hemopoiesis.

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Conflict of interest- None

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