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Full Length Research Paper

A study of cell passaging on mRNA expression, protein concentration and zymographic activity of MMP9

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The HL60 cell line, derived from acute promyelocytic leukemia cells, can differentiate into neutrophil-like cell following DMSO treatment. Mobility of HL60, or DMSO-differentiated HL60 cells (HL60), requires surface expression of adhesion molecules and production of matrix metalloproteinases (MMPs). The aim of this study was to investigate in HL60 and HL60 the effects of cell passaging (over 5 passages after delivery (P and P+5)) on i) surface expression of adhesion molecule CD11b, which is considered a neutrophil differentiation marker ii) MMP9 mRNA expression, protein release and zymographic activity and iii) cellular mobility. As expected, CD11b expression at both cell passages increased in HL60 relative to undifferentiated HL60, but expression levels of this neutrophils marker did not change over 5 passages. MMP9 mRNA expression however, in basal conditions was increased in HL60 at P+5. At P+5 versus P, MMP9 protein levels, MMP9 zymographic activity and cellular mobility in HL60 and HL60 were elevated. Stimulation by *N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine had no effects on HL60, but raised MMP9 protein concentration and zymographic activity in HL60. Since passage history is likely to also influence cellular functions other than MMP-related effects, it is important to carefully consider passage numbers when designing experiments.

Key words: Matrix metalloproteinases, mobility, cell passaging, HL60 cell line, DMSO-differentiation.

INTRODUCTION

The HL60 human leukemia cell line, derived from immune progenitor cells of a patient with acute myeloid leukemia, is characterized by a continuous proliferation in supension culture and by a capacity to differentiate *in vitro* to a number of different cell types such as mono-cytes or neutrophils (Collins et al., 1978). HL60 under-goes transition to neutrophil-like cells (HL60) after sti-mulation with a differentiation inducer such as dimethyl-sulfoxide (DMSO) and acquires some functions, surface markers and morphological properties of neutrophils (PN) which are essential for phagocytosis, killing of pathogenic bacteria and chemotaxis (Collins, 1987).

The invasive nature of neutrophils requires the ability to adhere to endothelial cells and to digest basement membrane components, like for example in cardiovascular complications such as myocardial tissue repair (Lindsey et al., 2001) . Firm adhesion of PN to the endothelial cells is mediated by the surface expression of adhesive mole-cules especially the integrin Mac1 which is consisted of the α-subunit (CD11b) and the β-subunit (CD18) (Wagner and Roth, 2000). Mac1 is involved in binding of neutro-phils to activated endothelium in most models of inflam-matory response (Luscinskas and Lawler, 1994; Malik and Lo, 1996). Adhesion is followed by migration of the PN into the site of inflammation (Roman, 1996; Springer, 1995; Tsai, 1998) and by production of matrix metallo-proteinases (MMPs), a family of proteolytic enzymes (Shi et al., 1999; Zucker, 1988) among which MMP9 was pre-viously depicted to play a major role for the digestion of basement

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Abbreviations: HL60, HL60 cell line; **HL60**, Neutrophil-like DMSO-differentiated HL60 cells; **DMSO**, Dimethylsulfoxide; **fMLF,** *N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine ; **MMPs**, Matrix metalloproteinases; **PN**, Polynuclear neutrophil.

membrane type IV collagen (Bernhard et al., 1990; Kahari and Saarialho-Kere, 1997).

During subculturing, modifications of phenotypical or functional characteristics of HL60 such as respiratory burst or secretion of β -glucuronidase have been described (Hadjokas et al., 1992). Previous observations in our laboratory suggest that secretion of superoxide anion by HL60, induced by addition of the bacterial peptide *N*-formyl-L -Methionyl-L-Leucyl-L- Phenylalanine (fMLF), a ligand inducing chemotaxis and superoxide production in neutrophils (Prossnitz et al., 1993), already decreases within fifteen subculture passages (unpublished results). In order to optimize the functional characteristics of HL60 as a model of neutrophils, we examined whether cell culture passaging is affecting their mobility and MMP9 characteristics. Further, surface expression of adhesion molecule CD11b, highly expressed in PN and used as a marker for differentiated neutrophils (Trayner et al., 1998), was measured in HL60 and DMSO-diffe-rentiated HL60. The consequences of cell passaging were studied on mRNA expression, protein concentration and zymographic activity of MMP9, as well as on migration and invasion properties of HL60 and HL60, stimu-lated or not by the bacterial peptide fMLF.

MATERIALS AND METHODS

Reagents

All chemicals were from MERCK (Overijse, Belgium), unless stated otherwise.

Cell cultures

HL60 cells (ATCC-LGC PROMOCHEM, batch 3641816, Manassas, USA) were used at passage P and P+5 after delivery and were grown in RPMI medium (CAMBREX Bio Sciences, Verviers, Belgium) supplemented with 10% heat-inactivated fœtal bovine serum, streptomycin (10 μg/ml) and penicillin (100 units/ml), at 37°C in a humidified air atmosphere containing 5% CO_2. Cultures of HL60 cells were maintained at a cell concentration between 0.2 x 10^6 and 1 x 10^6 cells/ml: 5 x 10^6 cells were seeded in 75 cm^2 culture flasks with 25 ml of culture medium. After 4 days the number of HL60 cells reached 25 x 10^6 (i.e. a concentration of 1 x 10^6 cells/ml) and were diluted (passaged) . To induce their neutrophil-like differentiation, HL60 cells were cultured in presence of 1.3% (v/v) dimethylsulfoxide (SIGMA CHEMICAL Co, Bornem, Belgium) for 4 days as described previously (Bréchard et al., 2006; Bréchard et al., 2005). 1.3% DMSO-differentiated HL60 (HL60) were not pas-saged, they were originating from respective HL60 passages. Differentiating cells have, however, a slower growth rate and were seeded at a higher cell density (0.6 x 10^6 cells/ml). Then, both control and differentiated HL60 cells, cultured in parallel, reach the same final density (1 x 10^6 cells/ml) after 4 days and are used for experiments. For the experiments in 12- well plates, 0.5 x 10^6 cells/ml of viable cells were used: these were either resting or differentiated HL60 cells, at passage P or P+5, stimulated or not by *N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine, fMLF (SIGMA CHEMICAL Co).

ELISA

A commercial MMP9 colorimetric sandwich ELISA kit (RAYBIO-TECH, Norcross, U.S.A) was used to measure protein concentration of MMP9 in cell culture supernatants. The MMP9 ELISA kit is employing a primary antibody immobilized to the wells specific for human MMP9 and a secondary biotinylated anti-human MMP9 antibody. It measures both pro and active enzymes forms (ProMMP9 and MMP9) of the MMP9 protein.

Zymography

Zymography is an electrophoretic technique, where an enzymatic substrate is co- polymerized with the polyacrylamide gel, which is used to detect specific enzyme activity. Sodium dodecylsulfate (SDS) polyacrylamide gel (PAGE) zymography was performed using 10% SDS gel containing gelatin type A and B (5 mg/ml each, SIGMA CHEMICAL Co). Samples of cell culture supernatants were centrifuged at 100 x g for 5 min to remove any cells or debris and 20 μl were mixed with Laemmli sample buffer (1:2 dilution) without reducing agents or heating. Prestained SDS-Page broadrange molecular weight standard was purchased from BIORAD (Nazareth, Belgium) and control recombinant human MMP9 from R and D SYSTEMS (Abington, United Kingdom). The protein on the gel were renatured by exchanging the SDS with 2.5% triton X100 (3 x 15-min incubations). The gels were subsequently incubated over-night at 37°C in the refolding buffer (50 mM Tris-HCl, pH 7.4, 100 mM $CaCl_2$ and 0.05% Brij 35). Gels were then stained 30 min with Coomassie Brilliant Blue and destained with 25% methanol and 10% acetic acid. The staining of the gels reveals sites of gela-tinolytic proteolysis as white bands on a dark blue background. Densitometric analysis was performed using the Gel Pro Express 4.0 software (INTAS, Göttingen, Germany). Results are Integrated Optical Density values (IOD) expressed in $pixels^2$.

RNA isolation and Reverse Transcriptase Polymerase Chain Reaction

Total RNA isolation was performed using Invisorb Spin Cell RNA Mini Kit (INVITEK GmbH, Berlin, Germany). Total RNA concentrations and purity were determined by measuring OD_{260} and $OD_{260/280}$ ratio on a NanoDrop spectrophotometer (NANODROP TECHNOLOGIES, Rockland, DE). Reverse transcription (RT) was performed using ThermoScript™ RT-PCR System from *in vitrogen* (Merelbeke, Belgium): total RNA (350 ng) were reverse- transcribed in a final volume of 21 μl of the reaction mix described in the kit.

Real-time PCR

Real-time PCR was performed on a BIORAD iCycler iQ detection system. Products were from BIORAD and the real-time PCR reaction buffer included the following: 20 nM primers, 15 ng cDNA and iQ SYBR Green supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP, 50 u/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers). Sequences of designed primers (Eurogentec, Seraing, Belgium) used for PCR: the MMP9 primers used were 5'-TCTTCCCTGGAGACCTGAGA-3' (forward) and 5'-ACCCGAGTGTAACCATAGCG-3' (reverse); the β-actin primers used were 5'-TGACCCAGATCATGTTTGAGA-3' (forward) and 5'-AGTCCATCACGATGCCAGT- 3' (reverse). Real- time PCR cycling conditions were set as follows: 95°C for 3 min, 40 cycles at 95°C for 10 s and 60°C for 30 s. All sample were analysed in triplicate. The comparative cycle treshold (ΔΔCt) method was used to describe the change in expression of the target gene to the cor- responding control group. Relative gene expression levels were assessed with ΔΔCt method and expressed as $2^{-\Delta\Delta Ct}$ using βactin as an internal standard.

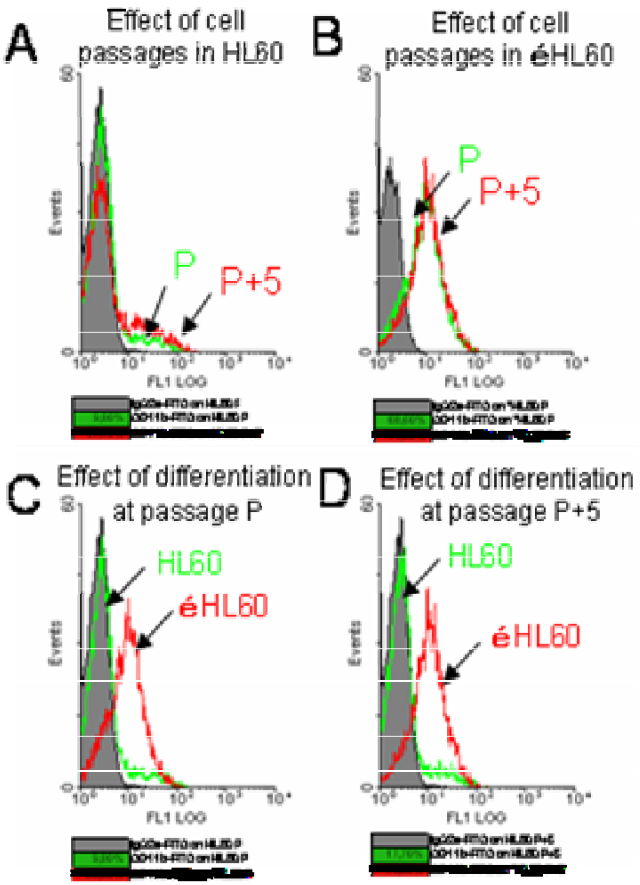

Figure 1. Flow cytometry analysis of CD11b surface expression expressed in percentage of positive cells: effect of cell passages (A and B) and effect of DMSO-differentiation (C and D) on CD11b surface expression on HL60 and HL60 at passage P and P+5. Graphs represent the number of cells (Events) as a function of fluorescence intensity (FL1 LOG). The gray areas show control fluorescence due to an unspecific antibody (IgG2a) for the studied cells. Results are percentages of CD11b-positive cells, measured with FITC-conjugated anti-CD11b antibodies at 488 nm. Representative experiment among at least 3 independent experiments.

Cell mobility: Migration and invasion assays

Migration and invasive properties of cells were quantified using the CHEMICON QCM 96 well migration (3 μm pore size membranes) and invasion (matrix coated 8 μm pore size membranes) assays (MILLIPORE-CHEMICON, Billerica, U.S.A) based on the Boyden chamber principle. For invasion assays, cells not only have to pass a porous membrane, but also ECMatrix™, a reconstituted basement membrane matrix of proteins derived from the Engelbreth Holm-Swam (EHS) mouse tumor. Wells were filled with medium with or without 10^{-8} M fMLF. The upper compartments were filled with 100 μl of a HL60 or HL60 cell suspension at 2 x 10^6 cells/ml. The assembled chamber (well + upper compartment) was incubated for 2 h (migration) or 6 h (invasion) at 37°C in a humidified air atmosphere containing 5% CO_2. Cells having moved towards the well were subsequently lysed and detected by the CyQuant GR dye™. This green-fluorescent dye exhibits strong fluorescent enhancement when bound to cellular nucleic acids. Quantification of fluorescence was performed on a Fluostar OPTIMA from (BMG LABTECH Gmbh, Offenburg, Germany) using a 480/520 nm filter set.

Flow cytometry

Monoclonal antibody labeling was conducted by a direct immune-fluorescence technique. HL60 and HL60 were first saturated with 5% purified human IgG (SIGMA CHEMICAL CO) in incubation buffer (137 mM NaCl, 5 mM KCl, 50 mM HEPES, 1 mg/ml glucose, pH 7.4) then labeled with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against surface marker CD11b or anti-IgG2a (IMMUNOTOOLS, Friesoythe, Germany). Cells (10^6) were incubated at 4°C for 30 min with FITC-conjugated anti -CD11b antibodies (dilution 1:10). Cells were washed once and re-suspended at 4 x 10^5 cells/ml in incubation buffer. Analyses of cells were performed on an Epics XL flow cytometer (BECKMAN-COULTER Inc, Fullerton, USA) with 488 nm filter. Results are expressed as percentage of positive fluorescence compared to an isotype control (IgG2a, negative staining).

Data analysis

Results are expressed as mean ± SEM. Significant differences (*,#: Pvalue<0.05) were calculated using one way ANOVA.

RESULTS

Effects of cell culture passages and fMLF on HL60 cells

Effect of cell culture passages: HL60 from P and P+5 were compared. First, we investi-gated surface expression of CD11b in basal conditions, as well as mRNA expression, protein secretion and zymographic activity of MMP9 or mobility of HL60, in basal and fMLF- stimulated conditions. FACS analysis showed that five cell passages did not modify CD11b surface expression in untreated HL60 (Figure 1A and Table 1). However, P+5 HL60 showed increased MMP9 mRNA expression, protein levels and zymographic acti-vities compared to P, in presence or absence of fMLF (Table 1 and Figure 2A). Mobility assays revealed that migration was not affected but that invasion of a coated membrane was increased at P+5, particularly in presence of fMLF (Table 1). Results show that most measured parameters increased in undifferentiated P+5 HL60.

Effect of fMLF

Addition of fMLF stimulates superoxide production, cell mobility (Prossnitz et al., 1993) and release of MMP9 in (Mackarel et al., 1999) neutrophils. In undifferentiated HL60, addition of fMLF neither modified expression of MMP9 mRNA at passages P and P+5 (1.7 ± 0.8 and 1.4 ± 0.3 fold in $2^{-\Delta\Delta Ct}$ vs basal condition, respectively), nor did fMLF modify the other studied parameters in HL60 (Table 1 and Figure 2A).

Table 1. Effects of cell passages on MMP9 mRNA expression, protein concentration and zymographic activity, on surface expression of CD11b and on migration and invasion properties in HL60 and HL60, under basal or fMLF-stimulated conditions.

	HL60		HL60	
	P	P+5	P	P+5
Basal				
MMP9 mRNA expression (vs P)	1.0	1.6 ± 0.1*	1.0	0.8 ± 0.3
MMP9 protein concentration (ng/mL)	1.9 ± 0.9	4.8 ± 0.1*	0.1 ± 0.1	1.6 ± 0.9
MMP9 zymographic activity (kpixels^2)	67 ± 28	256 ± 35*	17 ± 1	33 ± 9*
Migration (F.U.)	624 ± 101	865 ± 176	2252 ± 460	1861 ± 290
Invasion (F.U.)	108 ± 48	312 ± 73	102 ± 24	259 ± 23*
CD11b expression (% of positive cells)	9.9 ± 1.3	13.6 ± 2.9	47.9 ± 11.4	60.4 ± 7.1
fMLF				
MMP9 mRNA expression (vs P)	1.0	2.4 ± 0.2*	1.0	2.7 ± 1.2
MMP9 protein concentration (ng/mL)	2.0 ± 0.6	7.0 ± 1.4*	2.3 ± 0.6$^{\#}$	6.2 ± 1.0*$^{\#}$
MMP9 zymographic activity (kpixels^2)	78 ± 20	287 ± 20*	53 ± 1$^{\#}$	196 ± 42*$^{\#}$
Migration (F.U.)	612 ± 75	631 ± 92	2056 ± 311	1947 ± 276
Invasion (F.U.)	136 ± 41	325 ± 49*	89 ± 44	366 ± 59*

F.U. : fluorescence units. Results are means ± SEM of at least 3 independent experiments (*: significantly different from passage P or$^{\#}$ significantly different from corresponding basal condition, Pvalue<0.05).

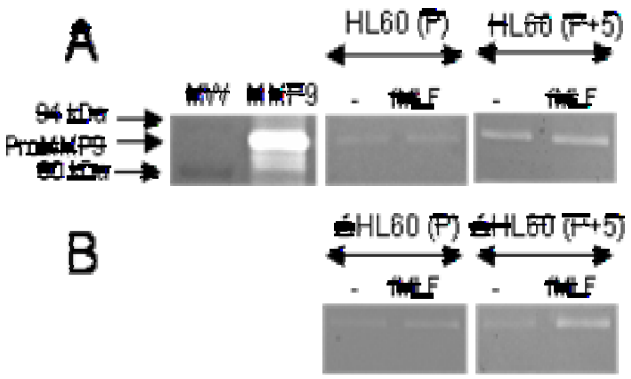

Figure 2. Zymographies: Effects of cell passaging on MMP9 zymographic activity in supernatants of HL60 (A) and HL60 (B), under basal or fMLF-stimulated conditions. MW: Molecular weight prestained SDS-Page standard broadrange markers, in kDa. MMP9: human recombinant control. Representative zymographies among at least 3 independent experiments.

Effects of cell culture passages and fMLF on HL60

Effect of cell culture passages

HL60 were driven into maturation by DMSO to model neutrophilic cells (HL60). Indeed, we observed an increased CD11b surface expression in HL60 at passage P and P+5 compared to the respective HL60 passages (Figures 1C and 1D, Table 1). Cell passaging did not affect surface expression of CD11b (Figure 1B and Table 1) and expression of MMP9 mRNAs (Table 1), but zymographic as did protein secretion (Table 1 and Figure 2B). Migratory function of HL60 was not modified, but their invasive activity increased at P+5, even further in presence of fMLF, ability increased at P+5 (Table 1). Results in Figure 1B and Table 1 show that similar to HL60, most measured parameters seem to increase in DMSO-differentiated HL60 cells.

Effect of fMLF

In HL60, fMLF did not modify expression of MMP9 at passage P and P+5 (0.6 ± 0.2 and 3.0 ± 2.0 fold in $2^{-\Delta\Delta Ct}$ vs basal condition, respectively) but it increased MMP9 protein release. Indeed, the effect of fMLF on MMP9 zymographic activity was around 2x more efficient at P+5 (Table 1 and Figure 2B). Invasion assays showed that fMLF seemed to stimulate invasive capacity of HL60 only at P+5 (Table 1).

DISCUSSION

HL60 cells differentiated by DMSO are capable of most neutrophilic functions such as chemotaxis, ingestion, respiratory burst and bacterial killing in response to ligands such as the bacterial peptide fMLF (Collins, 1987). Present results show significant biochemical and functional differences between HL60 and HL60, in relation to their subculture passage.

Cell lines can undergo undesired genotypic or phenotypic variations under serial subculturing process. A previous study shows for example ultrastructural variations in HL60 over a long subculturing period (100 or 200 passages) (Parmley et al., 1987). Functional impairments in β-glucuronidase secretion and respiratory burst are also depicted over 100 passages in HL60 (Hadjokas et al., 1992). Former

observations (unpublished results) in our laboratory however show that fMLF-induced superoxide production in our batches of HL60 substantially decreased already after fifteen passages. Therefore, we only used cells from pas-sages exhibiting high superoxide production. This obser-vation led us to investigate the influence of cell passaging on surface marker expression of CD11b, on MMP9 mRNA expression, protein release and zymographic activity, as well as on mobility of the neutrophilic model cells. The successful differentiation of HL60 into HL60 as a model of neutrophils was confirmed by a robust increase in CD11b surface expression, characteristic of neutrophilic differentiation (Trayner et al., 1998) . The expression levels of CD11b were not significantly affected by cell passaging, suggesting that a spontaneous differentiation of HL60, without addition of DMSO, as initially described by Collins (Collins et al., 1977) was not observed here after 5 passages.

However, in basal or in fMLF- conditions, cells showed an increase of MMP9 mRNA expres-sion (HL60), of MMP9 protein release and of cellular invasion properties (HL60 and HL60) at P+5. Previous studies report a decrease of MMP9 secretion related to a short period of 3 passages in human vein endothelial cells (Arkell and Jackson, 2003). Additionnaly, Kobayashi et al. (1998) show that levels of MMP2 decrease after sequential passages whereas MMP9 are detected at constant levels in keratinocytes (Kobayashi et al., 1998). Thus, different cell types exhibit distinct patterns of MMP9 release during cell passaging, suggesting that a delimited passage range should be used to study MMP9 characteristics. Furthermore, our data show that HL60 get more reactive to fMLF stimulation at higher passages: the effect of fMLF on MMP9 zymographic activity for exam-ple was approximately twice more important at P+5 compared to P, confirming that characteristics of these neutrophilic cells change in relation to cell culture passages. This higher sensibility to fMLF may also involve other metalloproteinases modifying functions like mobility, as indicated in our experiments by an enhance-ment of invasive capacity of HL60 and HL60 at P+5.

Conclusions

DMSO- differentiated neutrophil-like HL60 cells demonstrated passage-dependent effects on invasive capabilities and on MMP9 mRNA expression, protein concentration and zymographic activity. These results confirm that cell passaging differently affects cell lines functions, urging to check every cell batch for spontaneous variability for the parameters to be studied.

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