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PREFERENTIAL RELEASE OF HIGH AMOUNTS OF INTERLEUKIN-8 BY MYELOID BLASTS SHOWING MONOCYTIC DIFFERENTIATION

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ABSTRACT

Background. **In myeloid blasts, the expression and release of the multifunctional chemokine IL-8 could be expected to be differentiation-associated.**

Methods. **We investigated the profile of interleukin-8 (IL-8) expression and release by leukemic cells obtained at diagnosis from 42 untreated adult patients with acute myeloid leukemia of various FAB subtypes (2 M0, 7 M1, 6 M2, 6 M3, 10 M4 and 11 M5). IL-8 transcripts were evaluated by Northern blot and densitometric analysis. IL-8 release by myeloid blasts was evaluated by a specific ELISA either in sera at diagnosis or in supernatants (SN) obtained from cultured leukemic cells.**

Results. **In basal conditions, Northern blot analysis revealed detectable IL-8 transcripts in 15/29 cases, eleven of which were classified as M4-M5 and 4 as FAB M0-M3. Densitometric analysis of IL-8 transcript bands showed higher expression in M4-M5 than in M0-M3 cases (mean values ±SD: 16.5±21 and 0.77±1.36 densitometric units, respectively; $p=0.012$). Higher IL-8 serum levels were observed in leukemic patients as opposed to normal controls (mean values±SD: 0.53±0.75 vs 0.003±0.014 ng/mL, respectively; $p=0.006$). Furthermore, a trend (though not of statistical significance) towards higher IL-8 serum values was observed in M4-M5 as opposed to M0-M3 subtypes. After 24 hours of culture, the majority of myeloid blasts (95%) spontaneously released detectable amounts of IL-8 into SN. However, M4-M5 released substantially higher amounts of IL-8 than M0-M3 blasts (mean±SD: 68±46 and 8.5±12 ng/mL, respectively; $p<0.001$). This difference between M0-M3 and M4-M5 blasts was already observed after 6 hours of culture and increased over 72 hours.**

Conclusions. **Our findings confirm and further support the preferential release of high levels of IL-8 by myeloid blasts showing monocytic differentiation.**

Key words: IL-8, chemokines, AML, monocytic differentiation

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Interleukin-8 (IL-8) is a multifunctional chemokine that is a chemoattractant for neutrophils in inflammation, can influence the function of basophils and T lymphocytes, and can act as an angiogenic, proliferative, autocrine and metastatic factor in given pathological contexts.[1-8] It is produced by a variety of normal and neoplastic cells[7-14] either in response to stimuli, including interleukin-1 (IL-1), tumor necrosis factor-α (TNFα), lipopolysaccharide (LPS) and phorbol 12- myristate 13-acetate (PMA), or constitutively.[7,8,12,15-17]

IL-8 is also produced by mononuclear bone marrow cells,[17] human acute myeloid leukemia (AML) cell lines[18,19] and *ex vivo* human AML blasts.[20-22] These last produce IL-8 constitutively,[20-22] express IL-8 receptors,[21] and show an increase of IL-8 mRNA after short-term culture in the absence of any exogenous stimulus, suggesting that the machinery involved in IL-8 production in myeloid blasts is responsive to autocrine/paracrine regulation, possibly through an IL-1/TNF-dependent mechanism.[20]

The interest in studying IL-8 in AML is at least

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twofold. First, chemokines like IL-8 could play a role in conditioning blast-to-blast and blast-to-environment interactions and thus could be involved in deregulated mechanisms accounting for the biological and clinical behavior of AML blasts.[23-28] Second, IL-8 production by immature myeloid cells, both normal[17] and neoplastic,[20-22] suggests that IL-8 plays a part in myeloid differentiation. Data obtained in a previous study suggested a possible preferential expression and release of IL-8 by myeloid blasts with monocytic differentiation. In the present study we further investigated this issue in a larger series of cases, focusing our attention not only on IL-8 mRNA expression by leukemic cells in basal conditions, but also on IL-8 release into culture SN and on IL-8 serum concentration. Our data support the hypothesis that spontaneous release of high amounts of IL-8 in myeloid blasts is preferentially associated with monocytic differentiation.[20]

Materials and Methods

Patients

Leukemic cells were obtained from 42 untreated patients between 18 and 70 years of age with AML at diagnosis, referred to our Institution from 1989 to 1993. Most cases had a high white blood cell count (mean±SD×10^{-3} μL: 72.45±85.17) with a high percentage (>90%) of blasts and minimal residual contamination by normal cells. The diagnosis of AML was based on standard clinical, morphological, cytochemical, cytogenetic, and cytofluorimetric criteria. When needed, diagnosis was supported by bone marrow biopsy and immuno-histopathological assessment. Patients were classified according to FAB subgroups, as follows: 2 M0, 7 M1, 6 M2, 6 M3, 10 M4 and 11 M5.

Cell preparation

Blasts from freshly heparinized peripheral blood were separated by centrifugation on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and washed three times with PBS; cytospins and cytofluorimetric analysis (including evaluation of CD34, HLA-DR, CD33, CD13, CD14 and CD3, CD4, CD8) were performed to evaluate the characteristics of the blasts as well as the percentage of residual normal lymphoid cells. After separation, blast percentage accounted for 95-100% in all cases, the few normal cells being residual CD3$^+$ T lymphocytes. No residual neutrophil or normal-looking monocyte contamination was appreciable in any of samples. Cell viability was always >90%, as assessed by Trypan-blue staining.

Cell cultures

In each case 1×10^6/mL purified blasts/well were cultured for 24 hours and, in selected cases, for up to 72 hours in 24-well plates (Falcon, Lincoln Park, NJ, USA) in RPMI-1640 (Gibco Life Technologies Ltd, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco Life Technologies Ltd), penicillin (100 U/mL) and streptomycin (100 mg/mL), at 37°C in a 5% CO_2 atmosphere. According to the manufacturer, the final concentration of LPS in RPMI-1640 and FCS was 0.5 pg/mL and 1.6 pg/mL, respectively. Cultured blasts harvested after 10-minute centrifugation at 200 g were used for cytofluorimetric re-evaluation and cytospins. Cell viability was greater than 90%, as assessed by Trypan-blue staining. Culture supernatants (SN), collected at 24 hours in all cases and at 6, 24, 48, and 72 hours in randomly selected cases, were stored at –70°C until use.

Northern blot analysis

Total RNA preparation from purified uncultured blasts and Northern blot analysis (10 μg of RNA per lane) were carried out as previously described.[20] The RNA blots were hybridized to a ^{32}P-labelled cDNA probe for IL-8 (kindly provided by Dr. I. Lindley, Sandoz Institute, Vienna), and to a ^{32}P-labelled β-actin cDNA probe-containing plasmid (from Dr. R. Weinmann, Wistar Institute, Philadelphia, PA, USA). Bands on exposed films were scanned by laser densitometry and densitometric units (DU) were defined based on normalization of IL-8 to β-actin mRNA bands.

Detection of the IL-8 molecule in patient sera and culture supernatants

IL-8 molecule concentrations as ng/mL in

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cell-free culture SN and sera collected at diagnosis and stored at –70 °C until use were determined using a double-ligand ELISA (Bender MedSystems, Vienna, Austria), as previously described.[20] All samples were tested in duplicate and shared a variation coefficient (VC) <10%. RPMI-1640 medium alone and supplemented with 10% FCS, and 20 healthy blood donor sera were used as controls. Freezing procedures did not affect the measurements of IL-8, as assessed by appropriate tests.

Statistical analysis

Data are expressed as means±SD. The comparisons were performed with Student's t-test for unpaired data and considered significant if $p<0.01$.

Results

Table 1 summarizes the main clinical characteristics of the cases investigated, together with the pattern of IL-8 transcript expression, IL-8 production in SN and IL-8 serum concentration. In addition, Figure 1 shows the individual values of IL-8 mRNA expression in basal conditions, as evaluated by densitometric analysis of Northern blot bands in 29 cases grouped according to FAB subtypes. IL-8 transcripts in basal conditions were detected in 15/29 cases (52%), mostly (11/15, 73%) among M4-M5 cases. The amount of IL-8 mRNA was lower in M0-M3 than in M4-M5 blasts [DU: mean±SD 0.77±1.36 (range 0-4) in M0-M3 and 16.5±21 (range 0-61) in M4-M5] (p=0.012).

On the whole, IL-8 serum concentration was higher in leukemic patients (mean±SD: 0.53±0.75, range 0-3.07 ng/mL) than in healthy blood donor sera used as controls (0.003±0.014, range 0-0.06 ng/mL) (p=0.006). Although IL-8 serum concentrations were not significantly different in M0-M3 as compared to M4-M5 leukemias, a trend could be observed towards higher values in myelomonocytic subtypes (0.37±0.59, range 0-2.45 ng/mL, in M0-M3 vs 0.70±0.89, range 0-3.07, in M4-M5) (p=0.2).

As shown in Table 1 and Figure 2, SN concentration of IL-8 following 24-hour culture of leukemic cells was higher in M4-M5 (68±46, range 4.2-150 ng/mL) than in M1-M3 cases (8.5±12, range 0-40 ng/mL) (p<0.001). Although the majority of AML blasts (40/42, 95%) released detectable amounts of IL-8 into SN over 24 hours, IL-8 concentrations >10 ng/mL were found in 57% of leukemias, quite similar to the percentage of cases expressing IL-8 mRNA in basal conditions (52%). However,

Table 1. Main clinical characteristics and IL-8 pattern in 42 AML.

Patient	**FAB** subtype	**WBC** (10^{-3}/µL)	**IL-8** basal mRNA DU	in 24 h SN (ng/mL)	in sera (ng/mL)
1	M0	30	0	16	0.63
2	M0	111	0	1.8	0
3	M1	10	ND	4.2	0.15
4	M1	228	4	24	0.81
5	M1	22	ND	8.9	ND
6	M1	47	ND	24	0.27
7	M1	2.5	0	0	0.08
8	M1	57	ND	40	0.87
9	M1	30	0	1	0
10	M2	80	0	0.5	0.34
11	M2	65	ND	13.4	0.07
12	M2	18	0	0	0.14
13	M2	2.1	ND	5.2	ND
14	M2	5.8	ND	0.1	ND
15	M2	10	3	3.4	0.29
16	M3	1	ND	0.2	0
17	M3	1.4	1	33	0.44
18	M3	7	0	1	0.08
19	M3	150	2	1.7	2.45
20	M3	8	0	0.3	0.07
21	M3	111	0	0.05	0
22	M4	120	61	50	0.86
23	M4	66	0	115	1.42
24	M4	20	0	99	ND
25	M4	65	26	6	2.17
26	M4	400	ND	4.2	0.14
27	M4	8.2	ND	150	0.05
28	M4	99	ND	83.8	0
29	M4	30	0	80	0.5
30	M4	180	29	93	0.67
31	M4	22	1	13.4	1.32
32	M5	153	6	39	0.25
33	M5	34	6	93	0.22
34	M5	100	10	72	ND
35	M5	24	0	35	0
36	M5	142	39	128	ND
37	M5	33	ND	150	3.07
38	M5	20	20	53	ND
39	M5	30	ND	41.5	0.38
40	M5	50	0	8.9	0.15
41	M5	300	60	18	ND
42	M5	150	6	89.5	0

DU: Densitometric Units after normalization on the basis of the internal amounts of β-actin mRNA. SN: culture supernatant. Cultures were carried out in 10% FCS RPMI-1640. ND: not done. Mean±SD IL-8 values in 20 healthy blood donors: 0.003±0.014 ng/mL (range 0-0.06).

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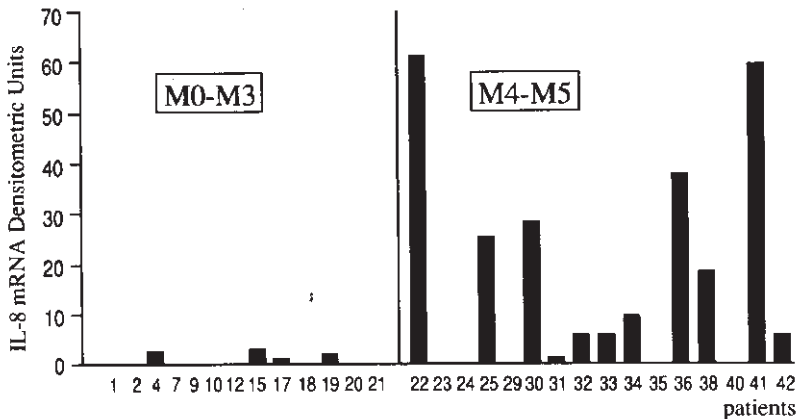

Figure 1. IL-8 mRNA levels in AML blast subtypes in basal conditions. The amounts of IL-8 mRNA are expressed as densitometric units after normalization on the basis of the internal amounts of β-actin mRNA. Patient numbers refer to Table 1.

among M0-M3 only 6/21 cases (29%) showed IL-8 SN concentrations >10 ng/mL, only 2/21 cases (9.5%) between 30 and 40 ng/mL (cases #8 and #17 in Table 1), and in no case were values >40 ng/mL observed. Conversely, among M4-M5 cases only 3/21 (14%) produced IL-8 SN concentrations <10 ng/mL, whereas the large majority (14/21, 67%) produced IL-8 SN concentrations >40 ng/mL.

Figure 3 shows a time course analysis (at 6, 24, 48 and 72 hours) of IL-8 release in SN by M0-M3 (4 cases) as opposed to M4-M5 (4 cases) blasts. The same Figure, left side, shows the pattern of IL-8 mRNA expression by the same cases in basal conditions. M4-M5 blasts are characterized by the release of higher amounts of IL-8; a clear-cut difference between M0-M3 and M4-M5 was already present after 6 hours of culture and was further enhanced up to 72 hours.

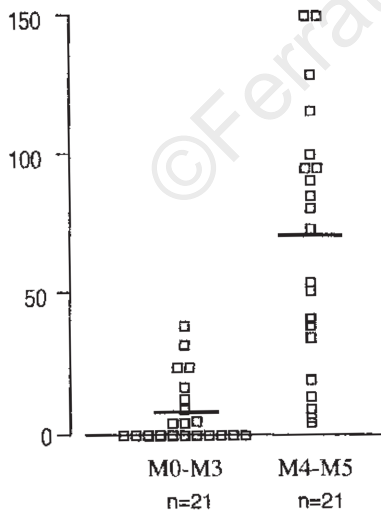

Figure 2. IL-8 concentrations in SN of 24-hour AML blast cultures in 10% FCS RPMI-1640. M0-M3 vs M4-M5 concentrations: $p<0.001$. RPMI-1640 medium alone and supplemented with 10% FCS were used as negative controls.

Discussion

Our study demonstrates that IL-8 is spontaneously produced and released, both *in vitro* and *in vivo*, by leukemic cells in the majority of AML cases. Although this phenomenon was not restricted to a given FAB subtype, it was preferentially observed in M4-M5 subtypes. In fact, the frequency and the amount of IL-8 transcripts were higher in uncultured blasts of M4-M5 as compared to M0-M3 cases. In addition, although detectable levels of IL-8 in culture SN were observed in the majority of AMLs (95%), their concentration was higher in M4-M5 than in M0-M3 cases (68±46 vs 8.5±12 ng/mL; $p<0.001$). In line with in vitro findings, IL-8 concentration in leukemia sera at diagnosis were higher than in normal controls and showed a trend towards higher values in M4-M5 as opposed to M0-M3 subtypes (though without a statistically significant difference).

These findings, which extend previous observations,[20,21] might be of biological and clinical relevance based on a number of considerations.

M4-M5s are clinically relevant AML subtypes accounting for up to 40% of cases. They usually present with aggressive clinical features, namely hyperleukocytosis and extramedullary tissue involvement, usually associated with poor prog-

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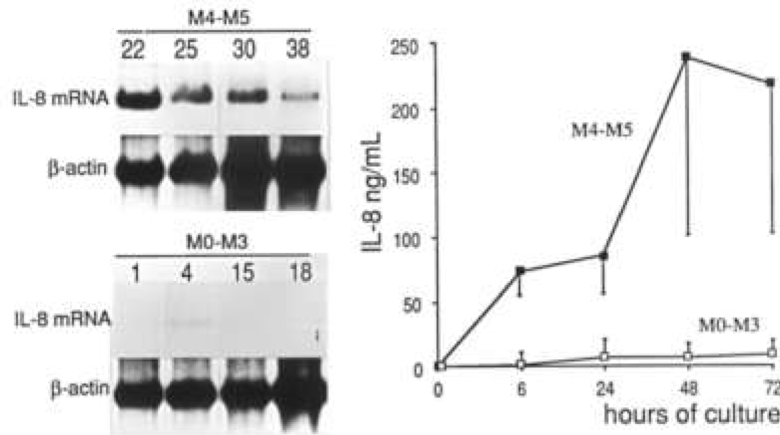

Figure 3. Basal expression of IL-8 mRNA and time course of IL-8 release in SN over 72-hour culture in 10% FCS RPMI-1640 observed in 4 M0-M3 as opposed to 4 M4-M5 cases. Left side: IL-8 mRNA expression in basal conditions by Northern blot. Numbers refer to patients in Table 1. Right side: mean amounts of IL-8 released by M4-M5 and M0-M3 blasts in SN at 6, 24, 48, and 72 hours. M0-M3 vs M4-M5 IL-8 concentrations in SN: $p<0.001$ at each time. RPMI-1640 medium alone and supplemented with 10% FCS were used as negative controls.

nosis. The preferential production of IL-8 by M4-M5 blasts might be regarded as a new facet in the biology of the aggressive group of monocytic leukemias.

Blast proliferation is influenced by signalling resulting from blast-to-blast interactions. For instance, a necessary condition for autonomous blast growth *in vitro* is that cultures must be performed at a density that permits cell interactions, even though intracellular loops exist which can support autonomous growth.[29] Moreover, normal tissue environments and blast cells can influence each other in a tissue-specific fashion through the effect of tissue-specific cytokines.[8,15,23] For example, IL-8 expression by melanoma cells is reported to be modulated by skin factors and to correlate with metastatic potential.[8] Due to their propensity for expressing IL-8, which facilitates cell-to-cell interactions and cell migration into tissues,[6,11] monocytic blasts can be expected to reach solid tissues more easily, which may result in local proliferation due to a synergism of blast- and microenvironment-released cytokines.[8,15,23] In skin, for example, keratinocytes can be induced to proliferate by IL-8 and can support an IL-1-rich environment,[8] allowing further local proliferative stimulation of blasts. In turn, extramedullary blast proliferation can contribute to frequent hyperleukocytosis in monocytic leukemias. This links the presence of high amounts of IL-8 to two relevant characteristics of acute monocytic leukemias, namely the involvement of extramedullary tissues and hyperleukocytosis. Clearly, such clinical characteristics represent the result of biological complexities which can hardly be related to the action of only one cytokine. Hence, the release of high levels of IL-8 by M4-M5 blasts should be regarded as a hallmark of the presence of a number of factors not yet fully characterized that are involved in regulating blast motility and migration.

The different pattern of IL-8 production by AML blasts with distinct differentiation features suggests a lineage-related tendency to produce IL-8 during early development, further supporting the concept that IL-8 might play a role in the early events of normal hemopoiesis. The induction of IL-8 production under LPS stimulation by mononuclear cells from normal bone marrow,[17] the down-regulatory effect of a number of cell differentiation agents on IL-8 production,[22,30] the expansion of the normal myeloid bone marrow compartment associated with leukocytosis, and extramedullary myelopoiesis in the spleen, liver and lymph nodes in mice lacking IL-8[31] are in keeping with this possibility. These findings suggest that IL-8 receptor, though not essential for hemopoietic development, for in mice lacking the IL-8 receptor all hemopoietic lineages eventually come to full maturation, could however be involved in the negative regulation of neutrophil production[31] and be relevant for an environmentally correct displacement and regulation of myeloid differentiation.

Thus the multifunctional chemokine IL-8 seems to play a part in events regulating bone marrow production and release of leukocytes

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and, to the extent that events in leukemia reflect normal hemopoiesis, IL-8 is likely to be involved in positive and negative regulation of the monocytic and granulocytic lineages, respectively.

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