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The cytokine-mediated crosstalk between primary human acute myeloid cells and mesenchymal stem cells alters the local cytokine network and the global gene expression profile of the mesenchymal cells

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ABSTRACT

Interactions between acute myeloid leukemia (AML) blasts and neighboring stromal cells are important for disease development and chemosensitivity. However, the molecular mechanisms involved in the cytokine-mediated crosstalk between mesenchymal stem cells (MSCs) and AML cells are largely unknown. Leukemic cells derived from 18 unselected AML patients were cultured with bone marrow MSCs derived from healthy donors; the populations then being separated by a semipermeable membrane. Coculture had only minor effects on MSC proliferation. The unique cytokine network in cocultures was determined by high constitutive MSC release of certain cytokines (especially IL-6 and vascular endothelial growth factor) and constitutive release of a wide range of soluble mediators by primary AML cells. However, the AML cell release varied considerably between patients, and these differences between patients were also reflected in the coculture levels even though supra-additive effects were seen for many mediators. These effects on the local cytokine network were dependent on a functional crosstalk between the two cell subsets. The crosstalk altered the global gene expression profile of the MSCs, especially expression of genes encoding proteins involved in downstream signaling from Toll like receptors, NFκB signaling and CCL/CXCL chemokine release. Thus, primary AML cells alter the functional phenotype of normal MSCs.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by bone marrow infiltration of immature leukemia blasts (Network, 2013; Welch et al., 2012; Reikvam et al., 2013a). The AML cell population has a hierarchical organization including a minority of leukemic stem cells (Bonnet & Dick, 1997) that often are included in the $CD34^+/CD38^-$ compartment (Bonnet & Dick, 1997). These cells show self-renewal and long-term in vitro proliferation and have the capacity to produce leukemic progenitors showing proliferative but not self-renewal capacity (Huntly & Gilliland, 2005). Both leukemic as well as normal hematopoietic stem cells (HSCs) depend on support from the bone marrow microenvironment (Lane et al., 2009). This support is at least partly mediated through the local cytokine network and the leukemic progenitors (i.e. the more mature leukemia cell subset) as well as the stromal cells contribute to this network through their constitutive cytokine release.

Mesenchymal stem cells (MSCs) can be isolated from various adult tissues (including bone marrow), they are multipotent and can differentiate into cells of the mesodermal (e.g. adipocytes, osteocytes, chondrocytes) as well as other embryonic lineages (Jiang et al., 2002). MSCs can also be cultured in vitro and retain their multilineage potential (Pittenger et al., 1999). It is not known whether AML cells can alter the biological functions of bone marrow MSCs. However, several observations suggest that cells with a stromal phenotype are important in leukemogenesis (Blau et al., 2011; Blau et al., 2007). MSCs may even mediate resistance against antileukemic chemotherapy (Kojima et al., 2011), possibly through their effects on the local cytokine network of the bone marrow (Bruserud et al., 2007; Uccelli et al., 2008). Finally, MSCs may also have immunomodulatory effects (Uccelli et al., 2008; Le Blanc et al., 2007; Le Blanc et al., 2008), but it is not known whether this is important for leukemogenesis or chemosensitivity. We have

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previously shown that the cytokine-mediated crosstalk between AML cells and differentiated stromal cells supports leukemia cell proliferation (Bruserud et al., 2004; Hatfield et al., 2006), and in the present study we investigated the cytokine-mediated crosstalk between primary human AML cells on immature MSCs.

2. Material and methods

2.1. AML patient population and preparation of primary AML cells

The study was approved by the local Ethics Committee (REK III, University of Bergen, Norway) and samples collected after written informed consent. Our department is responsible for the treatment of all AML patients in a defined geographic area, and the present study included 18 consecutive and thereby unselected patients (10 males and 8 females; median age 67 years with range 18–87 years) with high relative and/or absolute peripheral blood blast counts ($>7 \times 10^9$/L) (Supplementary Table 1). All patients were diagnosed according to the WHO criteria (Döhner et al., 2010), and most of them had *de novo* disease. All AML cell samples were obtained from peripheral blood, the leukemic cells were isolated by density gradient separation (Lymphoprep, Axis-Shield, Oslo, Norway) and gradient-separated cells contained at least 95% leukemic blasts. Cells were stored in liquid nitrogen until used in the experiments (Bruserud et al., 2003).

2.2. In vitro culture of mesenchymal stem cells and normal hematopoietic cells

2.2.1. Normal human MSC

Human MSCs derived from the bone marrow of healthy Caucasian donors (MSC24429, 45 years old male; MSC24539, 24 years old female; MSC25200, 38 years old male) were purchased from Lonza (Cambrex BioScience, Walkersville, MD, US). The cells showed no evidence for differentiation towards the adipogenic, chondrogenic and ostogenic lineages, the donors had no evidence for HIV-1, hepatitis B or hepatitis C infections, and all three cell preparations tested negative with regard to mycoplasma, bacterial and fungal infections (distributor's information). The MSCs were expanded in complete Mesenchymal Stem Cell Growth Medium (MSCGM™) (Lonza). Cells were obtained in passage two before being expanded, trypsinated and used for the experiments in passage 3–4. Viability determined by trypan blue exclusion was 50–80%..

2.2.2. Normal hematopoietic cells

Normal human hematopoietic progenitor cells (Lonza) were obtained by bone marrow aspiration from the posterior iliac crest of healthy donors. The cells were cultured for ten days in Poietics™ primary human hematopoietic progenitor growth medium that was supplemented with stem cell factor (SCF) 25 ng/mL, thrombopoietin (TPO) 50 ng/mL and FLT3-ligand (FLT3-L) 50 ng/mL. After culture 83.7% of the nucleated cells were $CD45^+/117^+/13^+/10^-/14^-$/HLA-$DR^+$ blast-like cells, 5.8% expressed lymphoid markers and 10.5% expressed neutrophil markers. The blast like population included 47% of cells showing low CD34 expression whereas the remaining 53% were highly positive for CD34.

2.2.3. Transwell cocultures

Coculture s of MSCs with normal or leukemic hematopoietic cells were prepared in six well plates (Costar 3401 transwell plates; Costar, Cambridge, MA, USA) where cells in the lower chamber are separated from the cells in the upper chamber by a semipermeable membrane (0.4 μm pore size). Cultures were prepared in complete MSCGM™ medium and MSCs were seeded in the lower chamber (6000 cells/cm^2). Pilot experiments showed that primary human AML cells cultured in this medium showed cytokine-dependent proliferation that was comparable to the response in the StemSpan SFEM medium (Stem Cells, Vancouver, Canada) (data not shown). After four days, 1×10^6 normal hematopoietic stem cells or primary human AML cells were added to the upper well. Cells were cocultured for three additional days before cells/supernatants were harvested or proliferation assayed by ^{3}H-thymidine incorporation as described in detail previously (Bruserud et al., 2004; Hatfield et al., 2006; Bruserud et al., 2003). The mesenchymal cells did not reach confluence during this culture.

2.2.4. Microtiter cultures of MSC

As described in detail previously (Bruserud et al., 2004; Hatfield et al., 2006) proliferation of MSCs was tested by ^{3}H-thymidine incorporation in microtiter cultures.

2.2.5. AML cell culture supernatants

In accordance with previous studies the AML cells (1×10^6/ml, 2 ml/well, 24 well culture plates) were cultured in Stem Span medium for 48 h before supernatants were harvested; at this time point the differences between patients with regard to ability of AML cells to show constitutive cytokine release are clearly seen (Bruserud et al., 2000).

2.3. Analyses of soluble mediator release

Supernatants were stored at −80 °C until analyzed. Cytokine levels determined by Luminex analyses (R&D Systems, Abingdon, UK) included (i) the growth factors tumor necrosis factor α (TNFα), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), heparin binding-epidermal growth factor (HB-EGF), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF); (ii) the interleukins (IL) IL1-RA, IL-1β, IL-6, IL-10 and IL-33; (iii) the chemokines CCL2-5, CXCL1/5/8/10/11; and (iv) matrix metalloproteases 1 (MMP-1) and 2 (MMP-2). IL-8/CXCL8, MMP-9, tissue inhibitor of MMP1 (TIMP-1) and angiopoietin-1 (Ang-1) levels were analyzed by enzyme-linked immunosorbent assays (ELISAs) (R&D Systems). All analyses were performed strictly according to the manufacturer's instructions.

2.4. Analysis of intracellular mediator levels

MSCs were trypsinized and the cell pellets (after centrifugation at 600 ×*g* for five minutes) were resuspended in Shieh lysis buffer. After 30 min of incubation on ice, the suspension was centrifuged at 13.000 ×*g* for 30 min. The clear lysate was harvested and stored at −80 °C until the levels of (1) p65 (RelA) that is the association partner of the mature subunit of NFκB, (2) the p65 inhibitor IκB, and (3) the p65 activator, IKKα/β were determined by Luminex analyses (Merck Millipore, Darmstadt, Germany).

2.5. RNA preparation, microarray analysis of global gene expression and PCR analyses

The MSCs from the lower wells were lysed in RLT plus buffer with added β-mercaptoethanol according to QIAgen's RNeasy protocol and thereafter homogenized by the aid of QIAshredder columns according to the manufacturer's recommendations. The lysates were stored frozen at −80 °C until thawing at 37 °C in a water bath and handled according to QIAgen's instructions in the RNeasy with DNase treatment protocol for the QIAcube. All microarray assays were performed using the Illumina iScan Reader and based on fluorescence detection of biotin-labeled cRNA. For each sample 300 ng total RNA was reversely transcribed, amplified and Biotin-16-UTP-labeled using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion; Foster City, CA, USA). Amount and quality of the biotin-labeled cRNA were controlled both by the NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer before 750 ng of biotin-labeled cRNA was hybridized to the HumanHT-12 v4 Expression BeadChip according to manufacturer's