Caspase-8 dependent apoptosis induction in malignant myeloid cells by TLR stimulation in the presence of IFN-alpha

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Abstract

Pro-apoptotic signalling upon toll-like receptor (TLR) stimulation in myeloid cells is normally antagonized by the simultaneous activation of anti-apoptotic pathways. We have previously reported that IFN-α can sensitize human monocytes to apoptosis induction by lipopolysaccharide (LPS). Based on these results we investigated whether similarly apoptosis can be cooperatively induced in myeloid tumor cells. When testing established acute myeloid leukemia (AML) cell lines we found the monocytic cell line THP-1 to be sensitive to IFN-α plus LPS induced apoptosis, which was partially dependent on caspase-8 and was associated with an enhanced expression of Fas/CD95. We extended our study to 29 short term blast lines from patients with AML and observed additive effects of IFN-α and LPS on cell death only with few samples indicating that sensitivity to IFN-α plus LPS inducible apoptosis is present in a fraction of AML samples only with no obvious correlation with certain FAB phenotypes.

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1. Introduction

The simultaneous targeting of separate cellular signalling pathways has great potential for improving the treatment of human malignant disease. There is great interest in developing therapy protocols with increased long-term effectiveness and reduced toxicity by utilizing synergistic effects of new drugs targeting signal transduction and/or influencing the regulation of cellular apoptosis. Along these lines, it has been reported that the inhibition of aberrantly activated protein phosphokinases resulted in the down-regulation of anti-apoptotic proteins and thereby effectively sensitized AML blasts to apoptosis induction by other agents [1–5]. Further downstream of the cellular signalling pathway, the down-modulation of NF-κB mediated anti-apoptotic gene transcription resulted in similar effects [6–11]. Other less well characterized synergistic combinations of cytotoxic agents have been reported [12].

We have previously found freshly isolated monocytes to be highly sensitive to apoptosis induction upon simultaneous stimulation by IFN-α and toll-like receptor (TLR) agonists such as LPS [13]. Signalling through TLRs in 293 cells, monocytes and macrophages has been reported by others to induce apoptosis after inhibiting NF-κB activation by additional agents [14,15]. IFN-α is a pleiotropic cytokine with different effects on tumor growth and differentiation and its effectiveness has been reported to be synergistically influenced by other agents [16]. In order to investigate our
observed synergistic apoptosis induction by TLR and IFN-α stimulation in more detail with the aim to elucidate the underlying mechanism, we performed experiments with established malignant myeloid cell lines and also primary AML blasts isolated from patients. In this paper we report apoptosis induction in the malignant cell line THP-1 by the combined action of IFN-α and LPS and found cell death to be associated with increased expression of Fas and to involve the activation of caspase-8.

2. Materials and methods

2.1. Cell culture

After having obtained informed consent, patient samples of peripheral blood were collected and mononuclear cells (MNCs) containing AML blasts were isolated by density gradient centrifugation using endotoxin-free Ficoll-Paque-PLUS® (Amersham Pharmacia Biotech AB, Uppsala, Sweden). HeLa and the myeloid cell lines HL-60, NB-4, OCI-AML5, MONO-MAC-6, MV4-11, KG-1a, U-937 and THP-1 were obtained from the German Resource Center for Biologic Material (DSMZ, Braunschweig, Germany) and were tested routinely for mycoplasma contamination during culture by MycoAlert detection kit (Cambrex, East Rutherford, NJ, USA). Mycoplasma positive cells were not used in any experiments. For apoptosis induction, the cell lines were cultured in RPMI 1640 (Invitrogen Corporation, Bethesda, MD) supplemented with 2 mM l-glutamine and 2% human serum (HS) (BioWhittaker, Walkersville, MD) and the AML blasts obtained from patients were cultured in RPMI 1640 supplemented with 10% of FBS (tested for <10 pg/ml endotoxin) (PromoCell GmbH, Heidelberg, Germany), 2 mM l-glutamine, 70.5 ng/ml G-CSF (Neupogen®, Amgen GmbH, Munich, Germany), 1000 U/ml GM-CSF (Leukine®, Berlex, Seattle, WA, USA), 10 ng/ml IL-3 (R&D Systems, Minneapolis, MN, USA), 10 ng/ml IL-6 (Strathmann Biotec, Hamburg, Germany), 10 ng/ml stem cell factor (SCF) and 50 ng/ml thrombopoietin (TPO) (PeproTech Inc., Rocky Hill, NJ, USA) as in the absence of these cytokines the viability of most patient blasts was strongly reduced. The cells were seeded at 0.2 × 10^6 cells/ml and analyzed after 3 or 4 days of incubation in presence or absence of 100 ng/ml lipopolysaccharide (LPS) (E. coli 055:B5; Sigma) and 1000 U/ml IFN-α2a (Roferon®-A; Roche, Basel, Switzerland). HeLa cells were used as positive control for caspase-8 activation in Western blot analysis and were incubated for 2 days in presence or absence of 100 ng/ml TRAIL (Alexis Corporation, Lausen, Switzerland). For inhibition of caspase-8 a cell-permeable irreversible peptide-substrate of caspase-8 (Merck Biosciences GmbH, Schwalbach, Germany) was added at 5 μM and 25 μM concentration 2 h before addition of IFN-α and LPS. A neutralizing monoclonal anti-FasL antibody (clone 2C101, Alexis) was added at 5 μg/ml for blocking the Fas/FasL interaction.

2.2. Flow cytometric analysis

Expression of CD95/Fas was determined using a monoclonal PE labeled CD95 antibody (clone DX2, Biolegend, San Diego, CA, USA) and a PE labeled IgG1 (clone DAK-GO1; Dako) as isotype control. The gate of analysis was set at propidium iodide negative cells. Cell death was quantified using FITC labeled Annexin-V (Resposif, Erlangen, Germany) and propidium iodide double staining as reported previously [13]. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data analysis was performed with CellQuest software (BD Biosciences).

2.3. Measurement of soluble FasL and TNF-alpha (TNF-α)

Human soluble FasL and TNF-α was quantified by standard sandwich ELISA methodology using a commercially available pair of monoclonal antibodies (Hölzel Diagnostika, Cologne, Germany).

2.4. Western blot analysis

Cells were collected, washed and lysed in 200 μl modified RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% IgePal CA-630, 0.25% Na-desoxycholate, 1 mM EDTA) supplemented with a protease inhibitor cocktail (BioCat GmbH, Heidelberg, Germany) by sonication for 20 s and incubation for 60 min on ice. Lysates were spun down and supernatants were stored at −80°C. A BCA protein assay (Perbio Science GmbH, Bonn, Germany) was performed and equivalent amounts of protein were resolved by 12% SDS-PAGE, transferred to a PVDF membrane (Carl Roth GmbH, Karlsruhe, Germany) and immunoblotted with anti-caspase-8 (clone 1C12, Cell Signalling Technology Inc., Danvers, MA, USA) and a horseradish peroxidase conjugated goat anti-mouse IgG1 secondary antibody followed by a visualization after exposure to X-ray films (Eastman Kodak, Rochester, NY, USA) using enhanced chemiluminescence (ECL, Amersham Biosciences, Freiburg, Germany).

3. Results

3.1. IFN-α plus LPS induces cell death in THP-1 cells

We have previously observed an exquisite apoptosis sensitivity of freshly isolated monocytes to low concentrations of LPS in the presence of IFN-α [13]. To test whether such an effect can be induced also in malignant myeloid cells we exposed the myelomonocytic cell line THP-1 to saturating concentrations of IFN-α, LPS, and IFN-α plus LPS. Fig. 1 depicts the size of the apoptotic fraction as determined by flow cytometry following Annexin V/propidium...
iodide staining after 3 days of incubation. IFN-α alone triggered apoptosis in 34.5% of THP-1 cells, and in combination with LPS 54.8% of the cells underwent cell death. LPS alone induced only little cell death (17.4%) perhaps reflecting its reported induction of anti-apoptotic NF-κB activity [17,18]. Besides THP-1, when we tested the myeloid cell lines HL-60, NB-4, U-937, MV4-11, MONO-MAC-6, OCI-AML5, and KG-1a we found only some anti-proliferative and/or apoptotic effects of IFN-α but no amplification with LPS (not shown). The marked response of myelomonocytic THP-1 cells to IFN-α/LPS but not of other cell lines of type FAB-M5 may point to the existence of escape mechanisms that can evolve in AML blasts and, together with our previous experience with peripheral blood monocytes, to a certain association of apoptosis sensitivity with monocytic differentiation. Still, the results obtained with THP-1 allowed us to perform further experiments with the aim to clarify mechanisms behind the established apoptosis induction.

3.2. IFN-α plus LPS upregulates the expression of fas and induces caspase-8 dependent apoptosis

LPS has been reported to increase the expression of Fas in THP-1 cells [19]. Interestingly, TNF-α in combination with IFN-α was found to strongly enhance Fas expression and to revert protective NF-κB activation into pro-apoptotic activity in colon carcinoma cells [20]. We therefore examined, whether IFN-α in combination with LPS would affect expression of Fas in a similar manner in THP-1 cells. Flow cytometry analysis revealed low constitutive Fas expression in untreated cells and cells treated with IFN-α alone (Fig. 2). LPS induced a marked upregulation of Fas in a small subpopulation of cells, which was strongly enlarged in combination with IFN-α.

Apoptosis can be initiated by the death-receptor (extrinsic) pathway and/or the mitochondrial (intrinsic) pathway [21]. Caspase-8 activation is the most upstream event of the death-receptor pathway, which often is amplified by participation of the mitochondrial apoptosis pathway. To explore the role of caspase-8 in IFN-α/LPS triggered cell death we exposed THP-1 cells to IFN-α/LPS in the presence of a cell-permeable and irreversible inhibitory peptide-substrate of caspase-8. As shown in Fig. 3, a high concentration of the inhibitor peptide (25 μM) clearly decreased IFN-α/LPS induced apoptosis in THP-1 cells during 3 days of culture. We next performed Western blot analysis to confirm the proteolytic activation of caspase-8 (Fig. 4). Part of the caspase-8 protein was apparently cleaved within 2 days of culture with IFN-α/LPS and also to a reduced extent with IFN-α alone, which closely correlated with the simultaneously measured proportions of Annexin V-positive cells (Fig. 4).
Fig. 3. Partial reduction of cell death in THP-1 cells by inhibition of caspase-8. A cell-permeable irreversible caspase-8 inhibitor peptide was added at the indicated concentrations to untreated THP-1 cells or 2 h prior to addition of IFN-α plus LPS. Annexin V positive cells were determined by flow cytometry after 3 days of culture in two independent experiments (shown as single data points).

### 3.3. IFN-α plus LPS induces cell death in blasts isolated from some AML patients

Based on the clear evidence obtained with THP-1 we asked, whether this mode of action of combined IFN-α plus LPS was active in AML blasts isolated from patient samples. We obtained 29 patient samples classified as FAB-M1 to FAB-M6 (M1: n = 5; M2: n = 4; M3: n = 5; M4: n = 8; M5: n = 6; M6: n = 1) and exposed them to IFN-α, LPS, and IFN-
α plus LPS during a short term culture over several days. The complete set of data is shown in Fig. 5, in which for better comparison the response of every donor is depicted as a line connecting the single values resulting from the four treatment conditions. Enhanced apoptosis induction by LPS plus IFN-α was seen only with one donor in the M2-subclass and with some patient samples of the M5-subclass. In two other cases LPS alone induced apoptosis and in about the half of cases some apoptosis was induced by IFN-α alone (Fig. 5). Generally, the viability and the responses varied between patient samples and did not show any specific pattern clearly associated with the different FAB classes of leukemia.

4. Discussion

We have previously found a strong induction of apoptosis by the combined action of IFN-α with LPS in peripheral blood monocytes [13]. We therefore asked in the present study, whether such an effect would also be active in malignant myeloid cells. Among established AML cell lines THP-1 displayed strong sensitivity to IFN-α plus LPS apoptosis which was at least partially dependent on caspase-8 activation and which was associated with an increased expression of Fas/CD95. When we examined patient AML blasts, we found similar apoptotic responses to IFN-α plus LPS combined stimulation, however, only in a minority of cell samples, perhaps indicating the frequent loss of components of the involved apoptosis pathways during malignant transformation.

Toll-like receptors similarly to TNF receptor I trigger anti-apoptotic and pro-apoptotic signalling pathways but normally the anti-apoptotic signals prevail in normal and malignant myeloid cells. LPS has been reported to induce anti-apoptotic c-IAP and FLIP protein expression [17,22] and to inhibit TNF-α induced apoptosis in U-937 cells [23], VP-16 induced apoptosis in THP-1 cells [18] as well as spontaneous Fas-mediated apoptosis in peripheral blood monocytes [22]. In our experiments we found low level apoptosis induction in THP-1 by LPS alone, which, however, was strongly enhanced by simultaneous action of IFN-α. A similar pro-apoptotic effect of IFN-α has been found in TNF-α and TRAIL-induced apoptosis in different malignant cells and has been explained by an inhibition of NF-κB by IFN-α [24,25]. NF-κB is constitutively activated and accounts for apoptosis protection in THP-1 cells [26] and it has been observed that signalling through TLR2 only after inhibition of NF-κB activation by proteasome inhibitors resulted in substantial apoptosis in THP-1 cells [14]. Similarly, apoptosis induction via TLR4 has been observed only after inhibition of NF-κB in murine macrophages [15]. Our data now suggest that signalling via TLR4 activates apoptosis pathways in human malignant AML cells resulting in rapid and extensive cell death in the presence of IFN-α, possibly due to IFN-α mediated interference of presumable simultaneously triggered anti-apoptotic signals.

Because of published evidence we investigated, if TNF-α and the Fas/FasL pathway are involved in the observed phenomenon and examined the influence of IFN-α and LPS on the expression of Fas, soluble FasL and TNF-α. TNF-α has been shown to trigger apoptosis in THP-1 cells [27] and in preliminary experiments we detected modest amounts of TNF-α (200–600 pg/ml) after stimulation with LPS and at very similar levels with LPS plus IFN-α (not shown). As LPS, however, can simultaneously inhibit TNF-α induced apoptosis [23], this might explain, why we found only little apoptosis with LPS alone. Regarding Fas, LPS has been found to enhance the expression of Fas in THP-1 cells. In our experiments this was confined to only a small subpopulation of the cells. On the other hand, LPS in combination with IFN-α strongly augmented the fraction of highly Fas positive cells. With regard to expression of FasL, we failed to detect soluble FasL at least within the detection limit of our assay (>50 pg/ml, limit of our assay, data not shown). However, monocytes and THP-1 cells have been previously established to contain preformed FasL, which upon stimulation was rapidly released, albeit at low levels [28], to trigger apoptosis [29,30]. Although THP-1 and other AML cells have been found to resist anti-Fas antibody triggering of apoptosis [31] we still considered the Fas/FasL pathway to be possibly involved as IFN-α has been reported to enhance Fas sensitivity in AML cells [32]. Such a mechanism was not supported, however, by blocking experiments using a neutralizing anti-FasL antibody, which could not diminish IFN-α/LPS induced apoptosis (not shown).

Our data show, that IFN-α and LPS cooperate to induce a partially caspase-8 dependent cell death. The activation of caspase-8 in THP-1 cells was clear but modest compared to that of HeLa control cells treated with TRAIL (Fig. 4). The relative contribution of caspase-8 to cell death in monocytic cells remains to be defined, but an additional participation of the mitochondrial or of further non-apoptotic pathways [33,34] seems likely considering the incomplete inhibition of IFN-α plus LPS induced cell death by the caspase-8 inhibitor peptide together with the only modest cleavage of caspase-8. On the other hand, our data provide evidence for the functional integrity of at least the caspase-8 pathway in THP-1. This is noteworthy, because functional blocks in caspase activation pathways are apparently common in AML [35,36] and therefore may also explain the only sporadic response of patient AML blasts to LPS plus IFN-α. Thus, although our study provides evidence that IFN-α and LPS synergize for cell death induction also in some aberrant myeloid cells, the majority of AML phenotypes might have unlocked these cooperative signalling pathways and require other mechanisms of sensitization.

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Contributions. W. Holter designed research and wrote the paper. O. Parolini was involved in project conception. M. Lehner wrote the paper and performed research. M. Bailo performed research. W. Roesler provided samples. D. Stachel provided samples and contributed conceptually. The authors reported no potential conflicts of interest.

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