Response of hairy cells to IFN-α involves induction of apoptosis through autocrine TNF-α and protection by adhesion

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Although hairy cell leukemia is uniquely sensitive to interferon-α (IFN-α), the biologic basis for this phenomenon remains unclear. Here we examine the effects of IFN-α on cultured hairy cells (HCs), taking into account the possible modifying influence of cell adhesion. We make the novel observation that therapeutic concentrations of IFN-α kill nonadherent HCs by inducing apoptosis. In keeping with the persistence of HCs in tissues during therapy, such killing was inhibited by integrin-mediated adhesion to vitronectin or fibronectin. Exposure of HCs to IFN-α resulted in a marked increase in tumor necrosis factor-α (TNF-α) secretion. Furthermore, blocking antibodies to TNFRI or TNF-RII protected HCs from IFN-α–induced apoptosis, demonstrating that such killing was mediated by TNF-α. In the absence of IFN-α, exogenous TNF-α did not induce HC apoptosis, showing that IFN-α sensitized HCs to the proapoptotic effect of autocrine TNF-α. This sensitization to TNF-α–induced killing was attributable to suppression of IAP (inhibitors of apoptosis) production known to be regulated by the cytoprotective nuclear factor-κB–dependent arm of TNF-α signaling. Moreover, engagement of the receptors for fibronectin or vitronectin prevented this IFN-α–induced down-regulation of IAPs. Understanding of the signals involved in the combined effects of IFN-α and TNF-α and abrogation of those induced by integrin engagement offers the possibility of sensitizing other malignant cells to IFN-α–induced killing and thereby extending the therapeutic use of this cytokine.

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Introduction

The response of hairy cell leukemia (HCL) to interferon-α (IFN-α) is one of the most dramatic and specific effects in the whole of clinical oncology. Nevertheless, the mechanism of action of IFN-α in HCL remains unclear. The agent inhibits HC proliferation induced in vitro,1,3 but there are no reports that it shortens the survival of these cells in culture. It is difficult to reconcile these observations with the fact that the clonal expansion in HCL, as in other chronic lymphoproliferative disorders, results mainly from prolonged cell survival rather than from increased proliferation.4 The aim of this study was to explore this issue further.

Among malignant B cells, HCs are distinctive in being constitutively highly activated cells and, in a number of previous studies, we have shown that this activation is responsible for many of the characteristic features of the disease.5,6 For example, HCs have constitutively activated adhesion molecules such as integrins, with the result that these cells readily interact with extracellular matrix (ECM) components without the need for exogenous stimulation.7 Such interactions are likely to modify the propensity of these cells to undergo apoptosis.

In HCL, treatment of patients with IFN-α induces rapid disappearance of HCs from blood. In contrast, the malignant cells remain in spleen and bone marrow for much longer periods and may not become completely eliminated from these tissues.8 Previous studies of the effects of IFN-α have shown that this cytokine increases autocrine TNF-α production and inhibits HC proliferation in response to cell stimulation.3 However, these observations do not explain the therapeutic effects of IFN-α, because TNF-α is described as an autocrine rescue factor for HCs9 and because HCL is a disease of prolonged cell survival rather than of increased proliferation.4

Therefore, a plausible explanation for the therapeutic effect of IFN-α in HCL is still lacking but is important for elucidating the specific biologic properties of HCs and for understanding why IFN-α has a therapeutic effect in only a limited number of malignancies. Here we examine how the combination of intrinsic and adhesion-generated signals influences the response of HCs to IFN-α. We show for the first time that IFN-α induces the apoptosis of HCs when they are deprived of the protective effects of cell adhesion. In contrast, IFN-α enhances the viability of chronic lymphocytic leukemia (CLL) cells cultured under identical conditions. We show that HC killing induced by IFN-α is mediated through a mechanism involving the up-regulation of autocrine TNF-α and sensitization of HCs to its proapoptotic effect via down-regulation of inhibitors of apoptosis (IAPs). Importantly, engagement of integrin receptors inhibits this latter effect, although it causes a further increase in the production of TNF-α. These observations not only shed light on the mechanism of action of IFN-α in HCL but also are of potential relevance for broadening the therapeutic applications of this cytokine.

Materials and methods

Patient samples

HCs were obtained from peripheral blood of patients with typical disease as determined by clinical presentation, malignant-cell morphology, tartrate-resistant acid phosphatase positivity, and immunophenotype. Cells from
iodide (DiOC 6 ) in phosphate-buffered saline (PBS) containing 1% BSA. Mononuclear cells were isolated from whole blood by centrifugation over Lymphoprep (Gibco, Paisley, United Kingdom) and, when more than 95% CD19+, were used without further purification. In some cases of HCL, contaminating T cells and any residual monocytes were removed by incubating the mononuclear cell fraction with monoclonal anti-CD3 and anti-CD11b followed by separation of antibody-coated cells with magnetic beads (Miltenyi Biotec, Surrey, United Kingdom); such further purification had no effect on any of the results obtained.

Reagents and antibodies

**Adhesive proteins.** Vitronectin (VN) was purified from normal plasma by heparin-Sepharose affinity chromatography according to the method of Yatohgo et al.13 The purity was determined to be more than 95% using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Coomassie blue staining. Fibronectin (FN) was purchased from Sigma (Dorset, United Kingdom).

**Antibodies and reagents.** The anti-CD95 antibodies (CH11 and ZB4) were purchased from Immunotech (Marseille, France), the anti-CD120a (H398) and anti-CD120b (MR2-1) from Serotec (Oxford, United Kingdom), and the anti–PARP-1 and anti-CD95 ligand (anti-CD95L) (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA). The anti–IAP-1 and anti–IAP-2 antibodies, the TNF–IAP-1 and PARP-1 antibodies, the TNF–IAP-2 antibodies, the TNF–anti–IAP-1 and anti-CD95 ligand (anti-CD95L) (C-20) were purchased from R&D Systems Europe (Oxon, United Kingdom). The respective class-specific and nonspecific polyclonal control antibodies were purchased from Becton Dickinson (San Jose, CA), and the IFN-α was from Wellcome (Beckenham, United Kingdom).

All other chemicals used were purchased from Sigma unless otherwise stated.

**Cell culture**

HCL or CLL cells (10⁶/mL) were cultured (37°C in 5% CO₂) in RPMI plus 0.5% bovine serum albumin (BSA). Culture vessels (Becton Dickinson) were precoated with VN or FN overnight at 4°C or with poly(2-hydroxyethyl methacrylate) (polyHEMA) for 48 hours at 37°C.

**Detection of cell death**

**Mitochondrial depolarization.** Cultured cells were gently resuspended and 200 μL added to an equal volume of 80 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC₆) in phosphate-buffered saline (PBS) containing 1% BSA. After 15 minutes of incubation at 37°C, an equal volume of propidium iodide (PI) (10 μg/mL) was added. After 30 minutes of incubation on ice, the cells were analyzed by flow cytometry. DiOC₆ is a cell-permeable green fluorochrome that is selectively concentrated within the polarized mitochondrial membrane (DiOC₆-dim/PI-dim [Figure 1A]), suggesting that the cation had no effect on any of the measured parameters.

**Results**

**IFN-α induces the apoptosis of HCs but not CLL cells.**

We first sought to establish whether or not cultured HCs could be killed by IFN-α. To take into account the possibility that cell adhesion might generate signals that inhibit IFN-α–induced killing, the tissue culture plastic was coated with polyHEMA (a nontoxic hydrophilic polymer that prevents cell adhesion). When HCs were cultured in this way, IFN-α induced progressive and concentration-dependent cell death as detected by mitochondrial depolarization (loss of staining with DiOC₆ [Figure 1A]) and by increased cell membrane permeability (increased staining with PI [Figure 1A]). Importantly, IFN-α produced a small increase in the proportion of cells with depolarized mitochondria and an intact cell membrane (DiOC₆-dim/PI-dim [Figure 1A]), suggesting that the cells were dying by apoptosis.14 To confirm that this was so, cells...
were examined for PARP-1 cleavage and DNA fragmentation because these events are specific to this mode of cell death. Exposure of HCs to IFN-α resulted in a marked increase in the number of cells with fragmented DNA (detected as a reduced DNA content following permeabilization [Figure 1B]). The cytokine also increased PARP-1 cleavage as detected by an increase in the ratio of p89 to full-length PARP-1 (Figure 1D). These findings indicate that IFN-α-induced killing was occurring, at least in part, by apoptosis.

We next sought to establish whether the proapoptotic effect of IFN-α in our culture system was specific to HCs. To do this, we examined the effect of IFN-α on the viability of tumor cells from patients with B-cell CLL, another mature B-lymphoproliferative disorder. In complete contrast to HCs, CLL cells cultured on polyHEMA were not killed by IFN-α; indeed, the agent enhanced cell survival to a variable extent in all cases tested (Figure 2).

**HC adhesion to VN or FN inhibits IFN-α-induced apoptosis**

To the best of our knowledge, IFN-α has not been shown in previous studies to induce HC apoptosis. However, such studies have usually included fetal calf serum (FCS) in the culture medium and have used plastic culture vessels that become coated with adhesive proteins such as VN and FN present in the FCS. Compared with HCs cultured on polyHEMA in BSA, HCs cultured in the FCS/untreated plastic system were indeed resistant to IFN-α-induced killing (Figure 3). This observation is likely to explain why the killing effect of IFN-α on HCs has not been observed in previous in vitro studies.

Previous work from this laboratory has shown that the ECM of spleen and bone marrow in patients with HCL contains substantial amounts of VN and FN, respectively,6,20 and that HCs interact with these proteins via specific integrin receptors.7 Because integrin engagement is capable of inhibiting apoptosis in other contexts,21 we postulated that the IFN-α–induced killing of HCs might be inhibited by integrin-mediated attachment of HCs to VN and/or FN. To examine this possibility, HCs were cultured on plates that were precoated with these ECM proteins or with BSA as a control surface. Measurement of cell death was performed by staining cells in situ with combinations of AO and EtBr and by Western blotting for PARP-1 cleavage. These methods were chosen because they enable both adherent and nonadherent cells to be analyzed.

As expected, IFN-α–treated HCs cultured on BSA underwent extensive apoptotic cell death as assessed by both methods (Figure 4). In contrast, HCs cultured on FN or VN underwent very little IFN-α–induced killing. This shows that IFN-α–induced apoptosis is inhibited by HC contact with these adhesive proteins.

To confirm that the antipapoptotic effect of FN and VN was mediated by integrin engagement, we examined the effect of
Moreover, in contrast to many other cell types, 28-31 HCs in the presence treated with IFN–/H9251, HCs rapidly disappear from blood but more spleen and bone marrow. This may explain why, in HCL patients adhesive proteins that have become part of the insoluble ECM of through interaction with ligand-coated plastic. Presumably, a effect on HC survival in the presence of IFN–/H9251 ligation (data not shown). Furthermore, a blocking CD95 mAb had no (Figure 6A), and IFN– did not sensitize them to killing by CD95–/H9251 (Figure 6C). Taken together, these results exclude an involvement of CD95/CD95L in the IFN––induced apoptosis of HCs. IFN– increases the production of TNF– by HCs and sensitizes them to the induction of apoptosis by this cytokine.

It has been reported that IFNs can also sensitize certain cells to other death-inducing agents such as TNF–. Moreover, IFN– is known to increase TNF– production by HCs, and IFNs sensitize monocytes to the induction of apoptosis by TNF–. Therefore, although TNF– is normally an autocrine survival factor for HCs, it is possible that IFN– may convert the effect of TNF– from an antiapoptotic to a proapoptotic one.

We first confirmed that TNF– production by HCs on both polyHEMA and VN is increased in the presence of IFN– (Figure 7). This increase was paralleled by an increase in TNF– messenger RNA. Thus, culture of HCs on polyHEMA in the presence of IFN– (100 U/mL) caused a 1.3-, 3.1-, and 4.5-fold increase in TNF– messenger RNA relative to the controls at 6 hours, 24 hours, and 48 hours, respectively (data not shown). By incubating IFN––treated HCs with blocking TNF receptor antibodies or control nonspecific antibodies, we tested whether the TNF– produced was responsible for induction of apoptosis by IFN–. Figure 8 shows that specific antireceptor antibodies inhibited IFN––induced cell death, with anti-TNF receptor I (CD120a) being slightly more effective than anti-TNF receptor II (CD120b) antibody. The presence of IFN– did not significantly alter the expression of either CD120a or CD120b (data not shown), indicating that changes in receptor expression levels were not responsible for HC killing. Furthermore, in the absence of IFN–, exogenously added TNF– did not induce the death of HCs cultured in suspension over an 8-day period, even when used at a concentration 100-fold greater than that found in supernatants of HCs cultured with IFN– (data not shown). This suggests that the increased TNF– production alone was not responsible for IFN––induced apoptosis. Taken together, the above data show that HC treatment with IFN– increases production of TNF– and, on a nonadherent surface, converts the TNF– action from a survival-promoting to a proapoptotic one.

To establish why comparable cell killing was not observed in adherent HCs, we then examined whether the rescue of these cells by VN from IFN––induced killing is mediated by inhibition of TNF– production. However, when HCs were cultured on VN, TNF– production was increased rather than decreased (Figure 7). Moreover, addition of IFN– to cells on VN caused a further increase in TNF– production (Figure 7) without marked induction of apoptosis. Thus, although TNF– production is important in the

**CD95/CD95L is not involved in IFN––induced apoptosis of HCs**

In other cell types, IFN– can up-regulate CD95 (Fas) and/or its ligand (CD95L), and may sensitize cells to CD95-induced killing. Moreover, both CD95 and its ligand expressed by a single cell type can influence cell survival by homotypic cell interaction.

We therefore examined the potential role of CD95/CD95L in the IFN––induced killing of HCs.

Here we confirm that HCs are strongly CD95 positive and show that CD95L expression is very low or negligible (Figure 6A). We also confirm that CD95 ligation does not induce HC apoptosis (Figure 6B), although such ligation induced killing of Jurkat cells (data not shown). Moreover, in contrast to many other cell types, HCs in the presence of IFN– failed to up-regulate either CD95 (data not shown) or its ligand (Figure 6A), and IFN– did not sensitize them to killing by CD95 ligation (data not shown). Furthermore, a blocking CD95 mAb had no effect on HC survival in the presence of IFN– (Figure 6C). Taken together, these results exclude an involvement of CD95/CD95L in the IFN––induced apoptosis of HCs. IFN– increases the production of TNF– by HCs and sensitizes them to the induction of apoptosis by this cytokine.

**Figure 4. Contact with immobilized VN or FN inhibits the IFN––induced apoptosis of HCs.** Cells were cultured as in Figure 1 except that the plates were precoated with VN or FN or with BSA as a control surface. After 6 days of culture, cell death was measured by double staining with AO and EB (A; dead cells appear red, while live cells are stained green) and by PARP–1 cleavage (B). A representative example of the 3 cases studied is shown. Original magnification × 100.

**Figure 5. GRGDS peptide prevents inhibition of IFN––induced killing by VN.** HCs were cultured for 6 days as in Figure 4 but in the presence or absence of GRGDS peptide (including 60 minutes of preincubation; 200 μM). Cell survival was measured by double staining with AO and EB. The results are from 2 identical experiments using cells from 2 different HCL patients.
apoptotic effect of IFN-α, modulation of the TNF-α effect by integrin signaling is able to inhibit the proapoptotic effect of IFN-α.

HC killing by IFN-α, and the rescue from this killing by adhesion, involves changes in IAP production

We next attempted to gain insight into the mechanism involved in HC killing by IFN-α and into how integrin signaling abrogates this killing. TNF-α may induce apoptosis via a pathway involving caspases and, at the same time, protect cells through activation of nuclear factor-κB (NF-κB). Transcriptional targets of NF-κB include cellular inhibitors of caspases known as IAPs. We have therefore measured NF-κB activation by electrophoretic mobility shift assay (EMSA) and IAP production, by Western blotting, in HCs in the presence or absence of IFN-α. Although we could not demonstrate an effect of IFN-α on NF-κB activation using EMSA (data not shown), under conditions of HC killing by INF-α (24-hour culture on polyHEMA) IAP-1 production was markedly reduced (Figure 9). In contrast, when cells were cultured on FN or VN this decrease in IAP-1 production was completely abrogated (Figure 9). Similar results were observed for IAP-2 (data not shown). Although EMSA did not demonstrate an effect of IFN-α on NF-κB activation, the observed decrease in IAP production is nevertheless likely to reflect an effect of this cytokine on the NF-κB pathway. Thus, it has been demonstrated that IFN-α can attenuate gene transcription by NFκB without altering its DNA-binding activity. The abrogation of IAP down-regulation by integrin signaling is also likely to reflect an effect on NF-κB because integrins are also known to activate the NF-κB pathway. Because the prevention of IAP down-regulation by adhesion to ECM proteins protected cells from apoptosis, this may explain why, during IFN-α treatment, HCs persist much longer within bone marrow and spleen than in peripheral blood.

Discussion

One of the most striking features of HCL is its uniquely high sensitivity to IFN-α. Nevertheless, despite considerable general knowledge concerning IFN-α-induced signals, the mechanism through which these signals specifically mediate the therapeutic effect of this cytokine in HCL is unclear. We have therefore examined the effects of IFN-α on HCs in vitro. It is well established that drug effects can be modulated by external microenvironmental influences such as adhesion and cytokines. Therefore, to eliminate such influences, we have cultured HCs on a nonadhesive surface (polyHEMA) and substituted BSA for FCS. Using this culture system, we now demonstrate the novel finding that IFN-α kills HCs through the induction of apoptosis.

To preserve cell viability, HCs have in the past been cultured using standard tissue culture plates in the presence of FCS. Our
results indicate that such a culture system rescues cells from IFN-α–induced apoptosis. Indeed, previous reports indicate that the presence of IFN-α under standard culture conditions only serves to limit HC proliferative potential. In our experiments, despite the absence of adhesion and FCS, good viability (> 80%) was maintained for up to 14 days of culture. When serum is present in the culture medium, HCs avidly adhere to the surface of the plastic vessel, which is known to become coated by serum proteins that include integrin ligands such as VN and FN. Cell binding to the surface-immobilized adhesive proteins results in the extensive integrin cross-linking required for optimal generation of survival signals. We therefore coated tissue culture plastic with purified VN or FN and showed that integrin engagement by these surface-immobilized proteins is sufficient to rescue HCs from the apoptotic effect of IFN-α. Although these proteins are present in the circulation in soluble form, they are abundant in tissues as part of the insoluble ECM of bone marrow and splenic red pulp where HCs accumulate and from where these cells disappear much more slowly during IFN-α therapy. We therefore propose that integrin receptor cross-linking by HC adhesion to ECM is responsible for the persistence of malignant cells in these organs during IFN-α therapy long after they have disappeared from the blood.

We next studied the mechanism of the observed IFN-α–induced apoptosis of HCs in the absence of environmental rescue. Several previous studies examining the killing of other cell types by IFN have implicated CD95/CD95L and by sensitization of HCs by IFN-α to the death-inducing effect of such autocrine TNF-α. Our novel observation that integrin engagement by adhesive proteins protects cells from the killing effects of combined IFN-α and TNF-α explains why in previous work where culture vessels become coated with such proteins, no killing of HCs by IFN-α was observed. In addition, our findings explain why during IFN-α therapy HCs disappear rapidly from the blood but persist much longer in spleen and bone marrow, where they are likely to be rescued by adhesion to ECM.

The challenge now is to elucidate the signaling basis of the modulation of the action of TNF-α by IFN-α resulting in cell killing and protection by integrin engagement from this killing. This could offer the possibility of using pharmacologic manipulation of relevant signals to extend the therapeutic use of IFN-α to malignancies that are currently resistant to the cytokine.

Because addition of exogenous TNF-α had no death-inducing effects in the absence of IFN-α, the killing of HCs by IFN-α could not be attributed simply to increased TNF-α production. Instead, our results indicate that IFN-α sensitizes HCs to the proapoptotic effect of TNF-α, a phenomenon that has also been observed in other cell types. We therefore examined the mechanism by which IFN-α and TNF-α cosignaling induces HC death and how integrin signaling protects HCs from this killing.

Cell stimulation by TNF-α can induce apoptosis through caspase activation, but this effect can be suppressed through the concomitant induction of IAP synthesis through the NF-κB pathway. Our study demonstrates that the induction of HC death by IFN-α involves suppression of IAP production and consequent sensitization of these cells to proapoptotic effects of TNF-α. Moreover, our experiments demonstrate that if integrin engagement by FN and VN inhibits IFN-α–induced killing of HCs and that the restoration of IAP production is involved in this cytoprotective effect.

In conclusion, the present study clarifies the mechanism of action of IFN-α in HCL. We show for the first time that malignant HCs, when deprived of adhesion, are killed by apoptosis in response to therapeutically relevant concentrations of IFN-α. This apoptosis is mediated by increased autocrine TNF-α production and by sensitization of HCs by IFN-α to the death-inducing effect of such autocrine TNF-α. Our novel observation that integrin engagement by adhesive proteins protects cells from the killing effects of combined IFN-α and TNF-α explains why in previous work where culture vessels become coated with such proteins, no killing of HCs by IFN-α was observed. In addition, our findings explain why during IFN-α therapy HCs disappear rapidly from the blood but persist much longer in spleen and bone marrow, where they are likely to be rescued by adhesion to ECM.
References


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