Differential release of chromatin-bound IL-1α discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation

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IL-1α, like IL-1β, possesses multiple inflammatory and immune properties. However, unlike IL-1β, the cytokine is present intracellularly in healthy tissues and is not actively secreted. Rather, IL-1α translocates to the nucleus and participates in transcription. Here we show that intracellular IL-1α is a chromatin-associated cytokine and highly dynamic in the nucleus of living cells. During apoptosis, IL-1α concentrates in dense nuclear foci, which markedly reduces its mobile nature. In apoptotic cells, IL-1α is retained within the chromatin fraction and is not released along with the cytoplasmic contents. To simulate the in vivo inflammatory response to cells undergoing different mechanisms of death, lysates of cells were embedded in Matrigel plugs and implanted into mice. Lysates from cells undergoing necrosis recruited cells of the myeloid lineage into the Matrigel, whereas lysates of necrotic cells lacking IL-1α failed to recruit an infiltrate. In contrast, lysates of cells undergoing apoptotic death were inactive. Cells infiltrating the Matrigel were due to low concentrations (20–50 pg) of the IL-1α precursor containing the receptor interacting C-terminal, whereas the N-terminal propiece containing the nuclear localization site failed to do so. When normal keratinocytes were subjected to hypoxia, the constitutive IL-1α precursor was released into the supernatant. Thus, after an ischemic event, the IL-1α precursor is released by hypoxic cells and incites an inflammatory response by recruiting myeloid cells into the area. Tissues surrounding the necrotic site also sustain damage from the myeloid cells. Nuclear trafficking and differential release during necrosis vs. apoptosis demonstrate that inflammation by IL-1α is tightly controlled.

hypoxia | necrosis | apoptosis | inflammation | alarmin

IL-1α and IL-1β affect inflammatory and immune responses, angiogenesis, and hematopoiesis (1, 2). The recombinant forms of IL-1α and IL-1β bind to the same receptor and exert similar biologic activities. Both IL-1α and IL-1β are first synthesized as precursor proteins; caspase-1 cleaves the inactive IL-1β precursor into an active cytokine, whereas the Ca^{2+}-activated protease calpain can cleave the IL-1α precursor into a mature 17-kDa form, liberating the 16-kDa N-terminal propiece cleavage product (ppIL-1α). However, constitutive IL-1α is rarely secreted from cells of mesenchymal origin, and epithelial cells, such as keratinocytes, leaving intracellular IL-1α in its full-length precursor form in these cells. In contrast, monocytes and macrophages do not contain preformed IL-1α but rather require de novo synthesis. After activation by calcium ionophores there is activation of membrane calpain, and the IL-1α precursor is processed and released. Indeed, fully active IL-1α precursor is present constitutively in epithelial cells, keratinocytes, and fibroblasts in healthy subjects, capable of triggering the IL-1RI (3). The N-terminal ppIL-1α contains a nuclear localization sequence and translocates to the nucleus, where it affects transcription (4, 5).

IL-1α belongs to an increasing number of "dual-function cytokines"; that is, cytokines that play a role in the nucleus, apart from their extracellular receptor-mediated effects. This group of cytokines presently comprises high-mobility group box-1 (HMGB1) (6–9), IL-33 (10), and IL-1α. Released as preformed cytokines, they can act rapidly in the immediate area and have been called "damage-associated molecular pattern" molecules (11–14). When cells die by necrosis (such as during injury, hypoxia, acidosis, or complement lysis), the intracellular contents are released, including cytokines in their precursor forms. These precursor forms bind and trigger their cognate receptors, resulting in a brisk inflammatory recruitment of infiltrating cells into the surrounding injured tissue. However, when programmed cell death takes place, apoptotic cells are ingested by macrophages without inflammation, thus preventing immune responses to cellular contents.

Therefore, a mechanism to inactivate biologically active molecules during apoptosis is required (15). Indeed, in the case of HMGB1, retention in apoptotic cells is via chromatin binding, whereas after cell necrosis HMGB1 is released into the extracellular compartment (6–9). The precursor form of IL-33 is also released from damaged cells and binds to its receptor ST2. However, after treatment with apoptotic agents, the IL-33 precursor is inactivated intracellularly by caspase-1 cleavage (16). Importantly, inflammatory responses to necrotic tissue in the absence of infection (i.e., ischemia) are uniquely dependent on IL-1 receptor (IL-1R) signaling and due to IL-1α, not Toll-like receptors (17, 18). Others have found that mesothelial cells respond in vitro to products of tissue necrosis with release of IL-1α (19).

In this study, we assessed the mechanism whereby IL-1α initiates inflammation depending on the type of cell death. We observed that similar to other dual-function cytokines, for example HMGB1 and IL-33, IL-1α is also a chromatin-associated nuclear factor. In addition, both precursor IL-1α and ppIL-1α are highly mobile proteins in living cells, but exhibit reduced mobility and are retained within the chromatin fraction during apoptosis. The potent biologic property of IL-1α was demonstrated by recruitment of myeloid cells in vivo and was restricted to IL-1α precursor form, containing both the N-terminal and the C-terminal receptor interacting domain. These data provide the link between intracellular IL-1α chromatin binding and its extracellular cytokine activity.

Results

Intracellular IL-1α Is a Chromatin-Associated Nuclear Factor. We examined whether IL-1α exhibits chromatin-binding properties

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enabling this duality of function by constructing a series of C-terminal GFP-conjugated clones of functional and mutated nuclear localization signal (NLS)-containing IL-1α molecules (Fig. 1*A*). B16 mouse melanoma cells were transfected with the described vectors and analyzed for IL-1α localization by confocal microscopy. In cells transfected with the mock GFP or the mutated NLS vectors, fluorescence was mainly concentrated in the cytosol, whereas precIL-1α and ppIL-1α rapidly translocated to the nucleus in transfected cells without any additional stimuli and were aligned with chromatin fluorescence (Fig. 1*B*). Within the cell, IL-1α is mainly present in its precursor form, which contains an active NLS in its N terminus and a C-terminal receptor interacting domain (20). We therefore focused on the precursor form, to further verify by ChIP assay the association of IL-1α with chromatin. Western blot analysis using anti-histone H3 antibody indeed confirmed the presence of chromatin in the ChIP precipitates of precIL-1α B16 transfectants (Fig. 1*C*). This evidence shows that IL-1α is a chromatin-associated protein. Thus, chromatin-binding activity of HMGB1, IL-1α, and IL-33 is a fundamental characteristic of these dual-function cytokines.

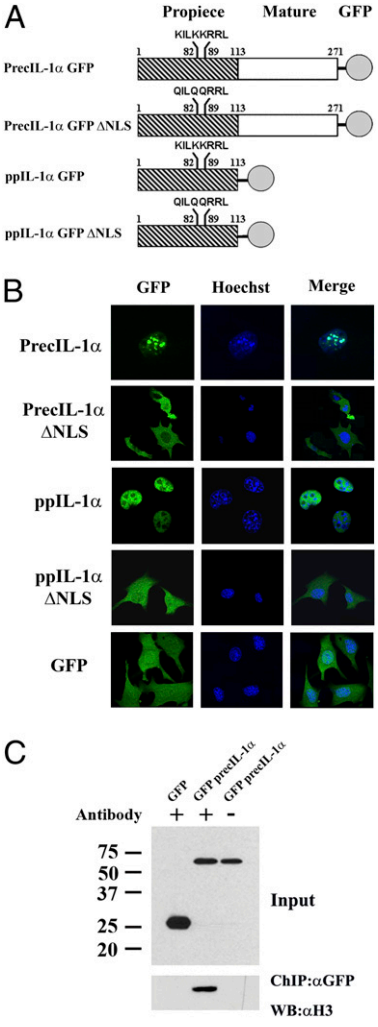

Fig. 1. IL-1α is a chromatin-associated cytokine. (*A*) Schematic representation of IL-1α GFP-conjugated proteins. The N-terminal domain containing the NLS in position 82–89 is denoted by a dark gray box. The C-terminal mature component (known as the IL-1R interacting domain) is represented by a clear box. (*B*) B16 melanoma cells were transfected with precIL-1α, ppIL-1α, and IL-1α mutants containing inactive NLS or GFP. Twenty-four hours later, nuclear localization and chromatin alignment were analyzed by confocal microscopy. (*C*) ChIP assays were performed using sheared chromatin from lysates of precIL-1α or GFP (as a control) transfected cells and were immunoprecipitated with anti-GFP antibody. An additional control assay was performed without the presence of antibody (beads only). The immunoprecipitates were separated over a 4–20% gradient SDS/PAGE gel and transferred to a nitrocellulose membrane. To verify chromatin presence, histone H3 antibody served as a molecular marker in Western blot analysis.

Chromatin-Associated IL-1α Proteins Are Highly Mobile in Living Cells and Exhibit Reduced Mobility During Apoptosis. We next examined IL-1α protein mobility within the nucleus of live and apoptotic cells by fluorescence recovery after photobleaching (FRAP) (21, 22). In living cells, the recovery of the photobleached nucleoplasmic area was rapid in cells transfected with constructs of ppIL-1α-GFP or precIL-1α-GFP fusion proteins, approaching prebleach levels within 5 s. This indicates that either form of IL-1α is highly mobile in the nucleus. However, during apoptosis, the fluorescence of the GFP-conjugated proteins was concentrated in highly dense foci, and only marginal amounts were present in photobleached areas. This significantly lower mobility of the IL-1α molecules presumably occurs because of its tight association with chromatin (Fig. 2). The differential kinetics of IL-1α GFP proteins measured by FRAP was comparable when apoptosis was induced by UV irradiation or with etoposide (VP16), a topoisomerase II inhibitor (Fig. S1). We further examined whether the immobile status of IL-1α GFP proteins is due to histone hypoacetylation associated with apoptosis. Trichostatin A (TSA), a histone deacetylase inhibitor, was added to the cells undergoing apoptosis before the FRAP assay. TSA addition failed to alter the apoptotic nonreversible binding of IL-1α proteins, although it was previously described to affect HMGB1 mobility (Fig. S2*A*) (6).

In addition, by treating apoptotic cells with the reactive oxygen species (ROS) scavengers butylated hydrozyanisole (BHA) or *N*-acetylcysteine (NAC), we ruled out the possibility that cellular changes occurred as a result of ROS production (15, 23). Although BHA is known to reduce nuclear fragmentation and apoptotic body formation in UV-irradiated cells (24), none of the ROS scavengers had any effect on IL-1α protein mobility in apoptotic cells (Fig. S2*B*). We also could not detect posttranslational modifications within IL-1α that might affect the protein dynamics during apoptosis (Fig. S3), such as phosphorylation at specific serine sites (25) or acetylation of lysine residues (Fig. S4). Taken together, we show here that IL-1α, exhibits unique dynamics during apoptosis, a phenomenon that was only previously attributed to HMGB1.

IL-1α Is Released Together with Other Cell Components from Necrotizing Cells but Is Retained Within Apoptotic Cells. We next performed biochemical assays to assess whether the dynamics of nuclear IL-1α affects its release from dying cells. First, we confirmed that neither secretion nor processing of precIL-1α occurred in the B16-transfected cells (Fig. 3*A*). To mimic the release of cell components during necrosis or apoptosis, we harvested living or apoptotic cells and subsequently subjected the cells to three cycles of freeze–thawing followed by additional fractionation by centrifugation (*Materials and Methods*). We observed that in necrotic cells IL-1α was present in the supernatant together with other cell components, for example β-actin (Fig. 3*B*), whereas precIL-1α in apoptotic cells remained in the insoluble fraction that also contained the chromatin. IL-1α was not found in supernatants of apoptotic cells, in contrast to β-actin (Fig. 3*C*). Moreover, Nonidet-P40 treatment of the apoptotic cell pellet did not release IL-1α from the insoluble material (Fig. 3*D*).

To ensure that IL-1α specifically remained within the chromatin fraction of apoptotic cells, we examined the necrotic and apoptotic supernatants for the presence of other nuclear molecules. Two known components of the polycomb repressive complex 2 are known to be nuclear localized proteins: Suz12 (suppressor of zeste 12) and Ezh2 (enhancer of zeste homolog 2) (26, 27) were both

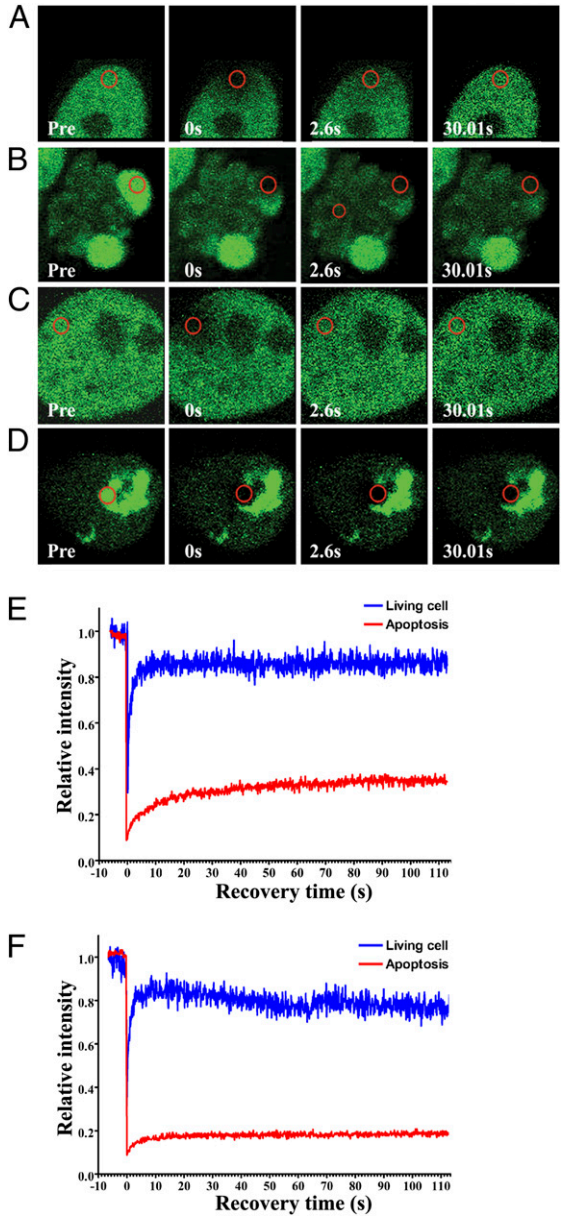

Fig. 2. Dynamics of IL-1α nuclear proteins in living and apoptotic cells. FRAP experiments in living and apoptotic cells expressing ppIL-1α-GFP and precIL-1α-GFP. (*A*) Bleached region of ppIL-1α-GFP–transfected B16 cell. (*B*) Bleached region of UV-irradiated ppIL-1α-GFP–transfected B16 cell. (*C*) Bleached region of precIL-1α-GFP–transfected B16 cell. (*D*) Bleached region of UV-irradiated precIL-1α-GFP–transfected B16 cell. Images were taken at the indicated times before (Pre), during (0s), and after the end of the bleach pulse (2.6s and 30.01s). The area of the bleach spot is indicated with a red circle. (*E*) Kinetics of nucleoplasmic FRAP in living or UV-irradiated apoptotic B16 melanoma cells transfected with ppIL-1α-GFP. (*F*) Kinetics of nucleoplasmic FRAP in living or UV-irradiated apoptotic B16 melanoma cells transfected with precIL-1α-GFP. Fluorescence intensity in the bleached region was measured for at least 110 s and expressed as the relative recovery. Data values in FRAP kinetics represent one of six independent experiments.

found in the soluble fraction obtained from necrotic and apoptotic cells, indicating that the apoptosis process per se does not induce general immobility of nuclear proteins (Fig. 3*E*).

Precursor of IL-1α Discriminates Between Necrotic and Apoptotic Cells with Recruitment of Neutrophils and Macrophages to the Site of Injury. To determine whether the differential release of IL-1α

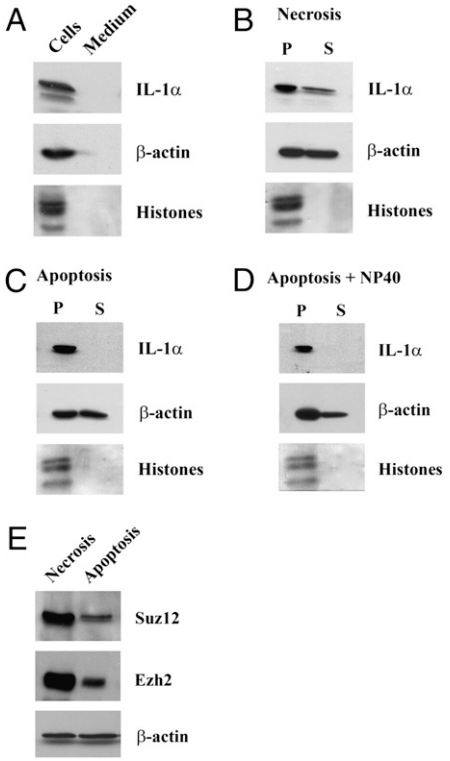

Fig. 3. Differential release of IL-1α by necrotic and apoptotic cells. The release of IL-1α and β-actin from precIL-1α-GFP–transfected B16 melanoma cells was determined by Western blot analysis, using the denoted antibodies. Histones were visualized using Ponceau S staining. (*A*) For the secretion of IL-1α and β-actin from intact live cells, the cells and the culture medium were analyzed by Western blot. Cell lysates from necrotic cells (*B*) or apoptotic cells (*C*) were obtained by three cycles of freeze–thawing and further fractionated by centrifugation. The soluble (S) and nonsoluble (P) fractions were analyzed as described above. (*D*) Apoptotic pellets were further treated with 1% Nonidet P-40 for 1 h, and both the soluble and nonsoluble fractions were analyzed. (*E*) Soluble fractions from necrotic and apoptotic (described in *B* and *C*) cells were analyzed for the presence of β-actin and the nuclear proteins Suz12 and Ezh2, using the appropriate antibodies.

from dying cells observed in necrosis vs. apoptosis affects a proinflammatory response, we used an in vivo animal model simulating the response to tissue injury. Supernatants of the apoptotic and necrotic cells (described above) were embedded into Matrigel and injected into mice, forming a 3D extracellular compartment or plug. Matrigel plugs provide a relatively defined environment enabling the isolation and characterization of infiltrating cells recruited by a particular stimulus. Thus, WT mice were injected with Matrigel mixed with supernatants of necrotic or apoptotic IL-1α transfectants. Matrigel plugs were removed after 20 h, and infiltrating cells were counted. Although both ppIL-1α and precIL-1α exhibit the same dynamics in the nucleus, only precIL-1α was able to recruit infiltrating cells, indicating that the C-terminal mature domain of IL-1α signals via the IL-1 surface receptor and is necessary for triggering an inflammatory response. Moreover, remarkably low concentrations of precIL-1α (20–50 pg embedded into Matrigel) from necrotic transfectants were sufficient to induce a robust infiltration of myeloid cells, demonstrating the high sensitivity of the surrounding tissue to IL-1α derived from necrotic cells (Fig. 4*A*).

Because IL-1α was not released from apoptotic cells, we next examined whether supernatants of apoptotic precIL-1α–transfected cells are able to trigger inflammation. As expected, the inflammatory response induced by lysates of apoptotic cells was dramatically reduced compared with that of necrotic cell supernatants (Fig. 4*B*). To further verify that the inflammatory response

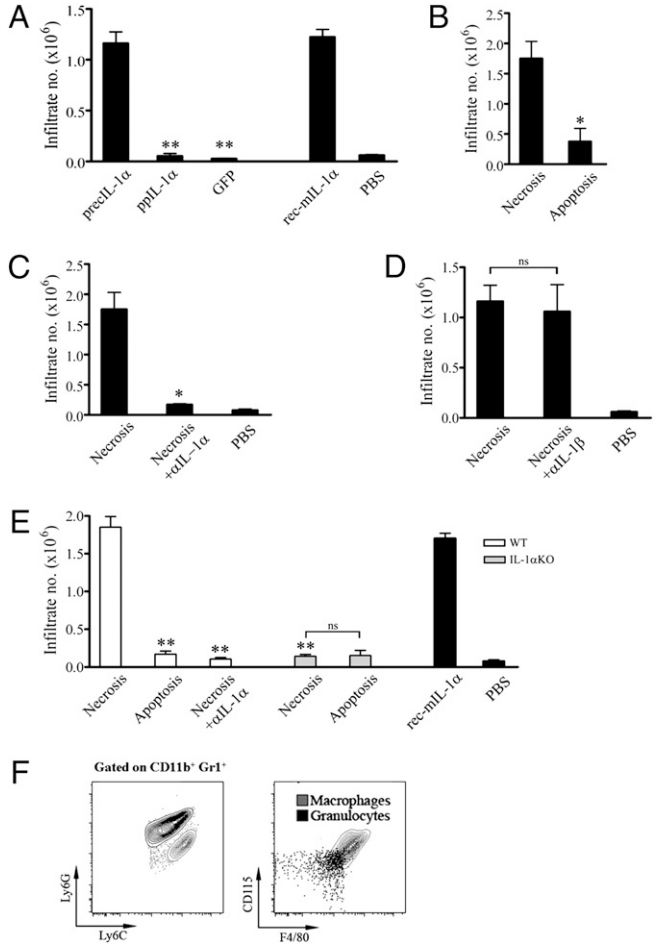

Fig. 4. IL-1α release from dying cells distinguishes between necrosis and apoptosis by triggering a proinflammatory response. (*A*) Number of infiltrating cells into Matrigel plugs 20 h after injection with supernatants of necrotic ppIL-1α, precIL-1α, or GFP-transfected B16 melanoma cells. Recombinant mIL-1α and PBS were used as positive and negative controls, respectively. $*P < 0.05$, $**P < 0.01$ vs. the precIL-1α group. (*B*) Infiltrating cell numbers in Matrigel plugs 20 h after injection with supernatants of necrotic and apoptotic precIL-1α–transfected B16 melanoma cells. $*P < 0.05$, $**P < 0.01$ vs. the necrosis group. (*C* and *D*) Infiltrating cell numbers in Matrigel plugs 20 h after injection with supernatants of necrotic precIL-1α–transfected B16 melanoma cells with or without neutralizing anti-IL-1α antibodies (*C*) or anti-IL-1β antibodies (*D*). $*P < 0.05$, $**P < 0.01$ vs. the necrosis group. (*E*) Necrotic and apoptotic WT and IL-1α$^{-/-}$ fibroblasts were injected with or without neutralizing anti-IL-1α antibodies together with Matrigel. Graph represents infiltrating cell numbers after 20 h. $*P < 0.05$, $**P < 0.01$ vs. the necrosis group. (*F*) FACS analysis of infiltrating cells into Matrigel plugs. Macrophages were defined as Ly-6C^{high}/CD115^{+}/F4-80^{+} cells; neutrophils were defined as Ly-6G ^{high}/Ly-6C^{dull}/CD115^{neg} cells. All experiments described in this figure were repeated three times, and each experimental group consisted of three mice. Data are from a single experiment and are presented as average ± SD.

induced by necrotic cells was IL-1α dependent, we neutralized necrotic cell lysates with specific antibodies. Anti-IL-1α antibodies inhibited the inflammatory response, whereas anti-IL-1β had no effect (Fig. 4 *C* and *D*), consistent with previous observations of Chen et al. (17).

To further provide evidence that this IL-1α–dependent response also occurs in nontransfected primary cells, we examined the inflammatory response induced by lysates obtained from mouse fibroblasts (Fig. 4*E*). Consistent with melanoma transfectants findings, lysates of necrotic WT fibroblasts induced a robust inflammatory response, whereas the apoptotic cell lysates recruited only small numbers of infiltrating cells. Moreover, necrotic fibroblasts obtained from IL-1α$^{-/-}$ mice generated a diminished response, similar to that induced by lysates from apoptotic IL-1α$^{-/-}$ and supernatants of necrotic cells neutralized with anti-IL-1α antibodies. We observed that most of the infiltrating cells were composed of macrophages and neutrophils, consisting of 50–70% of the total cell count (Fig. 4*F* and Fig. S5).

Precursor of IL-1α Is Released from Necrotizing Cells After Hypoxia. Many ischemic disorders (such as myocardial infarction, cerebral ischemia, and acute lung injury) result in necrosis due to acidosis accompanied by hypoxia. The inflammation in the rheumatoid arthritis joint is also hypoxic and acidic. In such cases IL-1α can be released from the cells into the surrounding tissue, initiating acute inflammation, particularly by recruitment of neutrophils and macrophages to the site. To demonstrate the release of IL-1α in ischemic tissue by a physiologic process, we subjected BD7 keratinocytes to hypoxia. BD7 cells were cultured in normoxic as well as hypoxic conditions for 24 h and evaluated by the numbers of necrotic cells due to the hypoxia conditions (Fig. 5*A*). Although the IL-1α precursor was present in both normoxic and hypoxic cells (Fig. 5*B*), increased levels of the cytokine were observed in the supernatants of hypoxic cells (Fig. 5*C*). These findings provide evidence that the function of IL-1α takes place under hypoxic and acidic conditions, resulting in necrosis, and likely contributes to the damaging consequences of ischemic disease.

Discussion

Whenever trauma or injury occurs, the immune system must make a clear distinction between pathologic and programmed cell death to promote an inflammatory response that will lead to tissue repair and not break tolerance by inducing autoreactive T cells. Thus, a vigorous inflammatory response should not be induced during programmed cell death (17). Intracellular molecules capable of being immediately released from injured cells alert the host of danger and are necessary for initiating the appropriate response. One major characteristic of these intracellular molecules is the ability to recruit leukocytes to the site of trauma. Two such molecules, characterized as endogenous danger signals released from damaged cells, are HMGB1 and IL-33 (8–10, 15). In the case of HMGB1, its release was shown to be prevented during the apoptotic process, whereas IL-33 was shown to be inactivated by caspase-1 cleavage, a process that is increased in apoptotic cells. It was not unexpected that constitutive IL-1α present in most cells in its precursor form induced an inflammatory response upon release by damage tissue (17). In tissue homeostasis, cells die by apoptosis and are ingested by macrophages, which concomitantly avoid an inflammatory phenotype, compatible with prevention of tissue damage. Upon tissue damage that occurs during hypoxia and acidic conditions, cell die of a necrotic process, releasing their contents and inducing a rapid inflammatory response, which damages the surrounding tissues in the short term but is also a prerequisite for tissue remodeling and repair.

Indeed, we have shown that in apoptotic cells, IL-1α remained associated with the chromatin and is not released into the extracellular compartment, therefore unable to induce inflammation. In contrast, upon cell necrosis, IL-1α is detached from the chromatin, released, and incites inflammation. This inflammatory response is mediated by the uncleaved IL-1α precursor, which is able to bind the IL-1R and initiate signaling. The ppIL-1α, which exhibits similar properties of chromatin binding and release upon cell death by necrosis, lacks the ability to interact with IL-1R and fails to induce inflammation. The infiltrating cells induced in situ by IL-1α released from necrotic cells consist of roughly equal numbers of neutrophils and macrophages.

The present study shows the unique aspect of IL-1α in the nucleus. Clearly, even in the steady-state condition, both the ppIL-1α as well as the precursor are highly mobile, an unexpected finding. The highly dynamic mobile nature of IL-1α in the nucleus provides

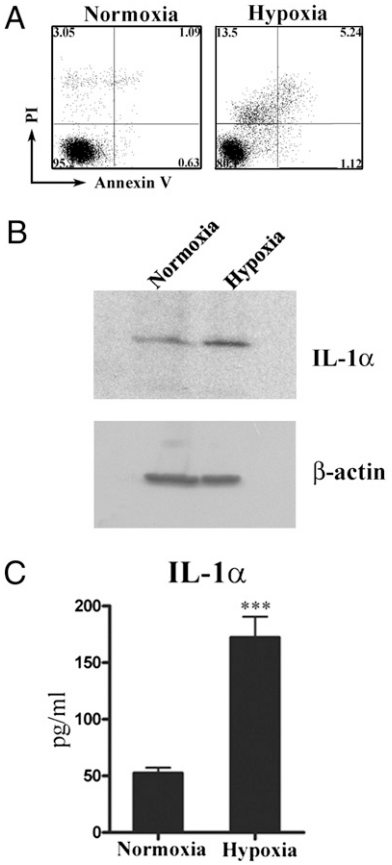

Fig. 5. The precursor of IL-1α is released from necrotic cells after hypoxia. (*A*) FACS analysis of annexin-V/PI–stained BD7 cells after 24 h culture in normoxic (*Left*) or hypoxic conditions (*Right*). (*B*) Lysates of the BD7 cells were analyzed by Western blot for IL-1α (*Top*) 24 h after culture in normoxic or hypoxic conditions, showing the 31-kDa precursor form. β-actin was used as loading control (*Bottom*). (*C*) Concentration of IL-1α in culture media of BD7 keratinocytes after 24 h culture in normoxic or hypoxic conditions, as measured by ELISA. Data represents the average ± SD ($n = 3$). ***$P = 0.0005$.

the cytokine with an alternative function for either mechanism of cell death. In the case of necrosis, little if any IL-1α remains associated with the nucleus, and the cytokine becomes part of the contents of the cell when released by loss of the integrity of the plasma membrane. The molecular mechanism by which IL-1α abandons the nucleus upon early signs of necrotic cell death remains unknown. In apoptosis an unknown mechanism effectively increases the binding of IL-1α to the nucleus, such that nearly all of the IL-1α is retained in the nucleus. The tight binding to chromatin is not due to histone acetylation: trichostatin A, an effective inhibitor of histone deacetylases, did not prevent IL-1α binding. Whatever accounts for the tight binding of IL-1α to the nucleus in cells undergoing apoptosis represents a physiologic mechanism to restrain inflammatory responses. Because cytokines such as IL-1α are essentially adjuvants for an immune response, the daily routine death of cells by apoptosis denies the immune system of a potential to break self-tolerance.

On the other hand, the release of the active IL-1α precursor from dying cells exacerbates the inflammatory responses by further cytokine secretion, especially by myeloid cells. In the lesson from the Matrigel studies, infiltrating macrophages are a potential source of IL-1β, and from previous studies, hypoxia stimulates the secretion of active IL-1β. The ability of the IL-1 receptor antagonist as well as specific antibodies to neutralize IL-1β in models of severe ischemia supports the concept that IL-1β is a major cause for sterile inflammation. How does one reconcile the findings of the present study indicating an apparently exclusive role for IL-1α in models of necrosis and hypoxia with the ability of antibodies to IL-1β to prevent damage in live animal models? Being preformed and released rapidly, the IL-1α precursor triggers the IL-1 receptor on adjacent cells and initiates the infiltration of myeloid cells. We have known for more than 20 years that IL-1α stimulates gene expression and release of active IL-1β from monocytes. Thus the initiator of tissue damage in models of ischemia seems to be due to the IL-1α precursor, but the amplifier of inflammation is likely IL-1β. The benefit of blocking IL-1β is during the amplification of the inflammatory response. Thus, although blocking IL-1β has been validated in animals as well as humans with ischemia, IL-1α is likely the initiator of sterile inflammation.

Materials and Methods

Cells and Constructs. IL-1α-pEGFP vectors were constructed by conventional PCR methods using mouse cDNA cloned into the pEGFP-N1 vector (Clontech) at 5′ HindIII and 3′ KpnI restriction sites. The site-directed mutagenesis approach was used to alter known serine phosphorylation sites at position 90 and 92 within the IL-1α precursor (26). The selected residues were replaced to alanine using the QuikChange XL site-directed mutagenesis kit (Stratagene). In all experiments, mouse B16 melanoma cells (that do not express IL-1α, as verified by ELISA) were transfected using the JetPEI (Polyplus Transfection) transfection reagent, and transfection efficiency was evaluated after 12 h using fluorescence microscopy and FACS analysis. WT and IL-1α$^{-/-}$ fibroblasts, both of C57BL/6 origin, were obtained as previously described (28).

For FRAP assays of IL-1α transfectants, apoptosis was induced by either 20 μg/mL VP16 (EBEWE Pharma) or UV irradiation for 3 min (50 J/m^2), and cells were analyzed by confocal microscopy after 6 and 8 h, respectively. For biochemical assays, 2.5×10^6 cells were plated in 10-cm Petri dishes, UV irradiated for 3 min to induce apoptosis, and maintained in fresh medium for 24 h. Apoptosis was evaluated by annexin-V/propidium iodide (PI) staining (Bender MedSystems) and confirmed to contain ≈90% apoptotic cells (Fig. S3). To obtain necrotic and apoptotic cell extracts, cells were harvested by centrifugation, washed three times with ice-cold PBS, and resuspended in 200 μL PBS at a cell density of $1–2 \times 10^7$/mL. The cells were then lysed by three cycles of freeze–thawing in liquid nitrogen and further fractionated by additional centrifugation at 12,000 × *g* for 10 min. For the detection of IL-1α-GFP (Santa Cruz Biotechnology), β-actin (MP Biomedicals), acetylated lysine (Santa Cruz Biotechnology), and nuclear factors of the polycomb complex Suz12 and Ezh2 (Cell Signaling), equal amounts of supernatants and cell pellets were analyzed by Western Blot analysis using the appropriate antibodies, and longer exposures were made to verify the absence of IL-1α.

BD7 cells were plated in six-well plates (10^6 in 800 μL medium per well), and hypoxic culture was performed in a sealed anaerobic workstation (Concept 400; Ruskinn Technology/Jouan), in which the hypoxic environment (O_2 <0.3%, 5% CO_2, 95% N_2), temperature (37 °C) and humidity (90%) were maintained. IL-1α from the normoxic and hypoxic cells was detected by Western blot using anti-IL-1α antibodies (R&D Systems) and in the culture media by ELISA (R&D Systems).

ChIP Assay. ChIP assays were performed using the EZ ChIP kit (Upstate Biotechnology), according to the manufacturer's instructions. Briefly, IL-1α and GFP transfectants were fixed with 1% formaldehyde for 10 min at 37 °C. To obtain fragments ranging from 200 to 700 bp, cells were sonicated five times for 20 s. To remove insoluble materials, the samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. Supernatants were incubated with or without anti-GFP antibody (Abcam, ab290) overnight, followed by the addition of protein A/G beads for 1 h. Beads were washed with low salt, high salt, LiCl buffers and twice with Tris–EDTA buffer. The beads were then boiled for 5 min, separated over a 4–20% gradient SDS/PAGE gel, and transferred to a nitrocellulose membrane. The presence of chromatin in the precipitants was verified using anti histone H3 antibody (Abcam).

Inflammation in Matrigel Plugs. Mice were injected s.c. in the interscapular area with 500 μL of 4 °C liquid Matrigel (BD Biosciences) (29, 30) mixed with 0.5 μg of necrotic or apoptotic supernatants (per mouse). As a control, Matrigel was premixed and injected with sterile PBS. For neutralization of IL-1α and IL-1β, 1 μg of specific neutralizing antibodies (R&D Systems) were added to the Matrigel mixture and incubated at 4 °C for 1 h before injection into mice. Matrigel plugs were surgically removed after 20 h and solubilized in 3 mL HBSS (GIBCO) containing 5 mg/mL collagenase type IV (Roche) for 1 h at 37 °C. Recovered infiltrating cells were washed three times in PBS and filtered through a 70-μm cell

strainer. The number of recovered infiltrating cells was determined by counting with a hemocytometer under light microscopy (×400), and cells were then used for FACS analysis. Single-cell suspensions obtained from Matrigel plugs were analyzed using flow cytometry (FACSCanto II; Becton Dickinson). Datasets were analyzed using FlowJo software (Tree Star). Monoclonal antibodies directly conjugated to FITC, PE, PE-Cy7, PE-Cy5.5, allophycocyanin, and pacific blue specific for the following antigens were used: CD11b (M1/70), Gr1 (RB6-8C5), CD45 (30-F11), F4/80 (BM8), CD115 (FMB,CSF-1R), and Ly-6C (AL-21), respectively (all from e-Bioscience). Cells were suspended in FACS buffer (PBS, 3% FCS, and 0.01% NaN_3). Dead cells were excluded by using the live/dead fixable aqua dead cell stain kit (Invitrogen).

Microscopy. Cells were plated and observed in Flourodish plates (World Precision Instruments). Live-cell microscopy was performed with an Olympus Fluoview FV1000 confocal microscope. Laser lines of 405 nm and 488 nm were used to observe Hoechst 33342 (Fluka) and GFP, respectively. For FRAP experiments, images were acquired at a rate of 5–7 frames per second. Fifty frames were acquired before bleaching. Selected areas were exposed to full laser power of a 488-nm line for 300 ms, followed by image acquisition for at least 110 s The time course of fluorescence intensity from the bleached area was extracted by Fluoview software and plotted using GraphPad Prism 4 (GraphPad Software).

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